ANTERIOR PITUITARY RESPONSIVENESS OF THE CYCLIC AND SEASONALLY ANOVULATORY MARE TO CONTINUOUS INFUSIONS OF GONADOTROPIN-RELEASING HORMONE

A Thesis

by

ISABEL CATALINA VELEZ JARAMILLO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2009

Major Subject: Physiology of Reproduction

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Approved by:

Co-Chairs of Committee, Gary L. Williams
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Committee Member, Katrin Hinrichs
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ABSTRACT

Anterior Pituitary Responsiveness of the Cyclic and Seasonally Anovulatory Mare to Continuous Infusions of Gonadotropin-Releasing Hormone.

(May 2009)

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In Experiment 1, 12 cyclic mares were assigned randomly to one of two groups (n = 6/group): 1) Control, saline; and 2) GnRH, 100 µg/h. Between 3 and 6 d after ovulation (Day 0), Alzet osmotic minipumps (Model 2ML1) containing saline or GnRH were placed subcutaneous and connected to a jugular infusion catheter. Five-min samples were collected from the intercavernous sinus (ICS) of 10/12 mares (5/group) during 8 h on Day 4, followed by an additional 6-h intensive sampling period 36 h after induced luteal regression (Day 6). Treatment with GnRH markedly increased ($P < 0.01$) secretion of LH during both luteal and follicular phases. During the luteal phase, treatment with GnRH eliminated the very large, intermittent secretory episodes of LH characteristic of controls and produced frequent episodes of LH release of short duration. In Experiment 2, 12 anovulatory mares and 3 mares with some residual follicular activity (n = 15) were used during the fall (December 5 to 20) and winter (February 15 to 29) seasons. Mares were assigned randomly to: 1) Control, 2) GnRH-20; continuous infusion

of GnRH at 20 µg/h, or 3) GnRH-100; continuous infusion of GnRH at 100 µg/h. Treatments were administered subcutaneously for 14 d using Alzet minipumps. Both the 20- and 100-µg/h treatments increased ($P < 0.01$) mean circulating concentrations of LH compared to controls before the winter solstice, but mares did not respond to the GnRH-20 dose after the winter solstice. GnRH-100 caused a seasonally-independent increase ($P < 0.0001$) in follicle size and ovulation frequency compared to controls The equine gonadotrope responded to continuous administration of high-dose GnRH during both ovulatory and anovulatory seasons, but was less responsive late compared to early in the anovulatory season.

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CHAPTER I

INTRODUCTION

The natural equine breeding season in the northern hemisphere is restricted to approximately the interval between April and October, with the majority of mares becoming anovulatory during the late fall and remaining anovulatory throughout the winter [1]. This 5 to 6-mo period of ovarian quiescence creates significant challenges for horse breeders involved with the > 40% of breed associations that have adopted January 1 as the universal birth date for their respective breeds.

Previous research has focused on developing a greater understanding of the neuroendocrine basis of equine seasonality, including the role of day length [1], pineal melatonin [2], and hypothalamic hormone secretion [3], as well as management strategies for obviating the effects of season. The control of photoperiod with artificial lighting is one managerial strategy that can be used effectively to hasten the onset of spring transition [4,5]. However, this approach is often associated with facility and cost-related limitations.

The administration of GnRH to induce follicular development and ovulation during the anovulatory season has also been investigated. Both pulsatile [6,7] and continuous [8] treatment with GnRH during late winter-early spring have been used with

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This thesis is written in the style and format of Theriogenology.

varying success. Clearly, pulsatile treatment with GnRH is more effective than continuous, and a consistent response has been observed following this treatment method between the months of January and April. However, there is evidence that the equine gonadotrope is relatively resistant to desensitization by continuous administration of native GnRH [9], and continuous infusion of doses ranging from as low as 2 μg/h [6,10,11] to as large as 110 μg/h have been successful in increasing the secretion of LH in mares during both the ovulatory [11] and anovulatory [6] seasons, as well as in ovariectomized mares [9]. Therefore, continuous treatment regimens have been explored more exhaustively for treating anovulatory mares than pulsatile treatment due to the practicality of the former approach.

Previous work conducted in this laboratory has shown that treatment of mares with low doses of GnRH (2.5-5.0 μg/h) beginning during late September/early October and continuing through March did not prevent the development of seasonal anovulation [12] or advance the timing of onset of spring ovulation, although the use of similar doses after April 1 was successful in approximately 80% of persistently anovulatory mares [11]. Therefore, the question remains as to whether the seasonal anovulatory state is controlled solely by a limitation of endogenous GnRH secretion, or, alternatively, involves changes associated with pituitary responsiveness. Additional studies in our laboratory utilizing the intercavernous sinus (ICS) sampling technique [13], that allows the measurement of pulsatile secretion of both GnRH and the gonadotropins, have found that GnRH concentrations do not change over the anovulatory season, therefore, resulting in the conclusion that GnRH may not be limiting during the anovulatory season. These

findings are contrary to accepted dogma which suggests that the lack of an adequate GnRH signal to the anterior pituitary is the primary cause of seasonal anovulation. Therefore, additional experiments are required in order to resolve this conflict and to more fully understand the unique interrelationships that appear to exist between the equine hypothalamus and anterior pituitary. The two approaches chosen for investigation in the current studies were designed to 1) better understand how the concentration patterns of ICS LH (gonadotrope response) change in response to continuous GnRH infusion during the breeding season, and 2) whether there are discernable changes in anterior pituitary responsiveness of anovulatory mares to GnRH before and after the winter solstice.

CHAPTER II

LITERATURE REVIEW

2.1 Endocrinology of seasonal reproduction

2.1.1 Common endocrine features of reproductive seasonality in mammals

In seasonal breeders, prolonged exposure to constant photoperiod induces photorefractoriness, causing spontaneous reversion in physiology to that of the previous photoperiodic state. In long-day breeders such as rodents, exposure to prolonged short photoperiod causes gonadal regression and the development of a winter phenotype; after a period of 12-20 weeks the animals progressively recover back to a summer physiological state. This mechanism allows long-day breeders in the natural winter environment to reactivate their reproductive axis in anticipation of spring, without the requirement for the stimulus of long photoperiod [14]. In the mare as in rodents, a short day length is associated with a marked decrease in gonadotropin secretion and consequently a decrease in ovarian activity [15]. In short-day breeders such as sheep and deer, this same mechanism is also present. Exposure to a fixed, long-day photoperiod causes refractoriness and reversion to a winter-like physiology and, when the photoperiod is held constant for a sufficiently long period, these animals express alternating transitions in seasonal physiology every 10 to 12 months [16].

Even though photoperiod and, consequently, melatonin production seem to have the principal roles in circadian rhythm, there are other factors that affect reproduction in

seasonal breeders: temperature, nutrition, body condition and ovarian steroids. In sheep, the seasonal changes in the pattern of circulating LH mainly reflect an increase in the brain responsiveness to the negative feedback exerted by estradiol during long days on the frequency of pulsatile secretion of GnRH and LH [17]. Moreover, the site of action of estradiol on the inhibition of LH secretion during anestrus in the ewe has been identified. Gallego-Sanchez and collaborators [18], after treating six different sites in the hypothalamus with intracerebral implantation of crystaline estradiol, determined that the retrochiasmatic area is the principal site of action of estradiol in the inhibition of LH secretion. On the other hand, treatment of mares with 1 mg of estradiol sc for 15 days during the nonbreeding season increases plasma LH concentrations [19]. However, during the breeding season, a negative feedback effect of estradiol seems operational and is necessary for the decline in LH after ovulation [1].

2.1.2 Role of the thyroid gland in seasonal breeding

Thyroid hormones are essential for the onset of anestrous in a spectrum of seasonal breeders. In sheep it has been demonstrated that the thyroid gland is essential in regulating transition into anestrous [20].

Frequency of LH pulses in thyroidectomized and thyroid-intact ewes is similar during the breeding season. However, circulating LH remained unabated in thyroidectomized ewes at the end of the breeding season compared to intact ewes in which pulsatile release of LH was inhibited [20]. On the other hand, Thrun and collaborators [21] have demonstrated that thyroid hormones are necessary only during a limited interval late in the breeding season to promote seasonal reproductive suppression in the ewe. They concluded that there is a critical period of responsiveness during which

thyroid hormones must be present for anestrous to develop. Replacement of thyroxine (T4) in thyroidectomized ewes implanted with estradiol during the later stages of the anestrous season demonstrated that ewes gradually lose responsiveness to T4 during the mid to late anestrous season. In contrast, the annual prolactin (PRL) cycle was not affected by thyroidectomy or T4 replacement, indicating that thyroid hormones are not required for all seasonal neuroendocrine cycles [22]. In another study, large doses of propylthiouracil (PTU) induced thyroid suppression and consequently appeared to inhibit onset of anestrus in the ewe [23]. Viguie et al [24] provided evidence that thyroid hormones may act within the brain to promote seasonal inhibition of neuroendocrine reproductive function of the ewe. Concentrations of LH declined in thyroidectomized ewes that were treated centrally with thyroxine while peripheral administration of the hormone failed to be effective for reducing concentrations of LH [24]. Contrary to the ewe, the roles of thyroid hormones in the mare have not been thoroughly investigated. Johnson et al [25] showed that thyroid hormones in the mare reach their greatest concentration in the circulation during the winter when the mare is typically anestrus. However, thyroidectomy failed to alter the onset of anestrus and, compared to ewes, thyroid hormones do not seem to play an important role in the control of seasonal reproduction in mares [26]. Further research is needed to fully understand the role of the thyroid gland in seasonal breeding of this species.

2.1.3 Hormonal and behavioral characteristics of reproductive seasonality

The estrous cycle of the mare averages 21 days and includes a period of recruitment of a new follicle wave stimulated by follicle stimulating hormone (FSH), followed by deviation, maturation and ovulation, stimulated by LH. Two or three

follicular waves occur during the estrous cycle that are each accompanied by an increase on FSH concentrations. At the end of the last follicular wave, the largest follicle(s), denoted as the dominant follicle(s), synthesize and secrete increasing amounts of estradiol, reaching a peak approximately 2 days before ovulation. Follicular-derived estradiol is positively correlated with intrafollicular concentrations of inhibin A and negatively correlated with FSH [27]. Estradiol induces estrous behavior which, in general, lasts 5 to 7 days in mares [28] and is a key factor for generating a full LH surge. Estradiol increases LH synthesis, induces GnRH receptors on gonadotrophs and may augment GnRH secretion [28].

The corpus hemorrhagicum at the site of ovulation becomes the CL, whose early development is regulated by LH. Progesterone (P4), produced by the CL, starts to increase in peripheral circulation soon after ovulation in the mare [29], while in the cow and sheep serum progesterone (P4) does not begin to rise until approximately 3 days after ovulation [1]. Progesterone is needed in order to maintain pregnancy and also promotes alveolar development in the mammary gland [1]. Luteal phase concentrations of P4 create negative feedback effects on LH [1]. On day 15 to 17 of the estrous cycle, the uterus releases $PGF_{2\alpha}$ in a pulsatile manner, associated with increase oxytocin (OT) blood concentrations; the increase in OT secretion is due to estrogen stimulation by the growing follicles [30]. This increase in $PGF_{2\alpha}$ causes luteal regression, which results in a decline in circulating progesterone concentrations to basal levels if regression is complete. Consequently, the negative feedback effect of P4 on LH is no longer present, resulting in a rise in that stimulates final maturation and ovulation of the new dominant follicle [1].

The natural breeding season of the mare terminates in October or November and is characterized by a failure to develop a follicle or to ovulate a developing large follicle [31]. Irvine and collaborators [32] assessed the secretion patterns of FSH in mares during the two ovulatory cycles before, and 24 days after, the last ovulation of the season. They also attempted to characterize hormonal profiles and follicular growth patterns with the goal of identifying changes that may lead to the termination of follicular growth and the breeding season. One observation was a change in the pattern of FSH release during the autumn transition from two surges to one surge per cycle. Moreover, LH secretion decreased as mares entered the anovulatory period. In another study [33], no differences in plasma concentrations of FSH were observed during the transition into the anovulatory season, nor were there any changes observed in the occurrence of the number of large follicles or in estrous behavior. However, concentrations of LH were lower following the last ovulation compared to the penultimate ovulation. Moreover, they also reported that some mares continued to show ovarian activity after the last ovulation, exhibiting follicular growth and estrous behavior [33].

The latter portion of the anovulatory season is associated with an increase in the number of follicles with a diameter of 15-25 mm and an increase in mitotic index of granulose cells [34]. A decrease in FSH concentrations and pulse amplitude has also been reported at this time [35] altough, this seems paradoxical since FSH is the hormone responsible for the increase in the mitotic index. For many mares, the ovulatory season is preceded by a period of prolonged “estrous-like behavior”, but these mares will often reject the stallion [36]. However, serum concentrations of LH do not increase until just before the first ovulation of the season [37].

2.1.4 Endogenous patterns of GnRH and gonadotropin secretion

The hypothalamic-hypophyseal axis serves as the master control center of reproduction in mammals. Gonadotropin-releasing hormone is the reproductive "master hormone" that is released from the hypothalamus and must reach the anterior pituitary through the hypophyseal portal vessels to stimulate gonadotropin (LH and FSH) secretion [38].

Some aspects of equine reproductive endocrinology are unique from that of other mammals. The content of GnRH in the hypothalamus and the quantity of GnRH receptors in the anterior pituitary seem not to change during the four seasons [38,39]. One study that contradicted these findings [40] reported the content of GnRH in the hypothalamus to be lower in the median eminence of seasonally anovulatory mares during one week before and 12 weeks following the winter solstice. Content of GnRH in the median eminence was increased on the day of the winter solstice.. Based on these results, one might conclude that the seasonal restoration of reproductive endocrine function in mares occurs more rapidly at the hypothalamic level than at the pituitary or ovarian level. It is well documented that, during the winter anovulatory period, circulating concentration of LH are very low and ovarian activity is limited [13,35,38]. However, one must be cautious in interpreting changes in hypothalamic function based on solely on changes in content of GnRH.

Another aspect of mare reproduction that differs from females of other species is that the pre-ovulatory LH surge is prolonged, with serum/plasma concentrations increasing gradually from mid-cycle until the day of the peak, approximately one day after ovulation [1]. The decrease in secretion of LH after ovulation appears to be driven

by ovarian negative feedback effects at the level of the pituitary and not by a down regulation of GnRH receptors on the pituitary as is thought to occur in other mammals [41].

Greaves et al. [42] studied the effects of ovarian inputs on GnRH and LH secretion immediately post-ovulation in pony mares. Blood samples were collected from the jugular vein and ICS on the day of ovulation and on day 8 post-ovulation in ovariectomized mares (OVX within 12 hours of ovulation) and intact mares; GnRH and LH concentrations were measured. Plasma concentrations of LH remained elevated in OVX mares, while plasma concentrations of LH declined in intact mares. There was a decline in secretion of GnRH from estrus to day 8, although there was no difference between the two groups. They concluded that the decline in LH requires ovarian negative feedback in mares.

Concentrations of hypothalamic GnRH and pituitary FSH and LH have been measured, although these measurements do not provide an assessment of the secretion of these hormones. Moreover, because GnRH is secreted in very low concentrations for local effects at the anterior pituitary, and because it is rapidly degraded in the blood, its relative mean concentration cannot be measured in peripheral plasma or serum. However, different techniques have been developed in an effort to overcome these disadvantages. Sharp and Grubaugh [43] utilized the technique of push-pull perfusion, which involves the placement of a concentric cannula into the area of the medial basal hypothalamus to perfuse a carrier medium through the tissue to estimate hypothalamic GnRH secretion in conscious horses. Secretory rate was found to increase progressively from anestrus to estrus in the breeding season. However, there was not a temporal

agreement between LH and GnRH pulses. Overall, there were about 65% as many peaks of LH identified as there were peaks of GnRH. Occasionally, a peak of GnRH was seen accompanied by a peak of LH. Therefore, the main objective of this experiment, to understand the neuroendocrine mechanism regulating gonadotropin secretion in the mare, was not completely achieved with this technique.

In 1984, Irvine and Hunn [44] developed the intercavernous sinus (ICS) cannulation technique for measuring both GnRH and the gonadotropins in the mare. Using this approach, the superficial facial vein is cannulated and the tip of the cannula is threaded into the ICS where venous effluent from the pituitary can be collected. This technique allows the collection of blood under complete physiological conditions without unduly disturbing the animal. Use of this method for measurement of pulsatile GnRH, LH and FSH secretion is possible only in equids due to the unique venous drainage of the pituitary and the arrangement of the equine cranial vasculature. Investigators showed that radiographs could be used to help overcome misplacement of the cannula and that chewing, changes in head position or vascular reaction of the cannula itself had no effect on the concentrations of the hormones on ICS effluent [45].

There are a few studies that have reported the use of this technique during various periods of the ovulatory and anovulatory seasons. Silvia et al. [46] determined pulsatile patterns of LH and FSH in the ICS and in peripheral jugular blood samples. They found no correlation between the pulsatile patterns of gonadotropins in the pituitary blood (ICS) and those in jugular blood during the follicular phase. Throughout the luteal phase, the frequencies of LH and FSH pulses in the ICS were 0.6 and 1.8 pulses in 8 h, respectively. During the follicular phase, frequencies increased to 7.4 and 6.8 pulses in 8 h,

respectively for each hormone. Pulses generated during the follicular phase were not detected in jugular blood, although the pulses detected during the luteal phase were recorded in peripheral plasma because of their greater amplitude [46]. Similar results were reported by Irvine and Alexander [47]. During mid-diestrus, a total of four peaks of LH and FSH were detected in a combined sampling period of 75 h in pituitary venous blood. These peaks were also detected in jugular blood and they were not associated with an immediate increase in progesterone concentrations in peripheral plasma. On the other hand, during the follicular phase, peak frequency was greater with 28 peaks of each gonadotropin in a combined sampling time of 80 h. Only 57% of pituitary venous LH and 50% of pituitary venous FSH peaks were detectable in jugular blood. Gonadotropin-releasing hormone was detected during each peak of gonadotropin secretion throughout the first sampling and 65% during the second sampling. They suggested that GnRH pulses might not be concurrent with gonadotropin pulses during the ovulatory surge because of the influence of high plasma estradiol creating a de-synchronization of the firing of GnRH neurons. In our laboratory, GnRH, LH and FSH were measured during four different seasons. No difference in FSH mean concentrations nor amplitude of pulses were detected among all seasons; however, there was a slight but statistically significant decrease observed in frequency of pulses of FSH during fall transition compared to all other seasons. Mean concentrations of LH and amplitude of LH pulses were greatly reduced during fall transition, anovulatory season and early ovulatory season compare to the ovulatory season. However, contrary to the accepted dogma, neither the frequency of LH pulses, nor the frequency and amplitude of GnRH pulses changed

between seasons [13]. Furthermore, the mechanisms regulating the decrease in LH during anovulatory season remain to be elucidated.

2.2 Neuropeptide regulation of seasonality

2.2.1 Role of melatonin in seasonal breeding

Photoreceptors in the eyes respond to light excitation and provide signals to the pineal gland through retinohypothalamic and suprachiasmatic neurons that modify the synthesis and secretion of melatonin. The nocturnal duration of melatonin secretion is greatest during fall and winter months when nights are the longest. Evidence from one study with mares indicated that melatonin reduces GnRH content of the hypothalamus [48], suggesting that gonadotropes may lack adequate stimulation to synthesize and secrete LH during the winter anovulatory period. Moreover, the action of melatonin is mediated by specific high-affinity, membrane-bound receptors that inhibits GnRH-induced calcium release from the endoplasmic reticulum as well as calcium influx through voltage-channels in the gonadotrops. Melatonin also inhibits GnRH-induced accumulation of cAMP in the gonadotrops , which may result in the decreased influx of calcium, because cAMP, acting through protein kinase A, stimulates calcium influx into the gonadotrophs [49]. Fitzgerald and Schmidt [2] found that, during the winter anovulatory season, concentrations of melatonin in plasma increased during the night time in both non-cycling and cycling mares, although, for some individuals, a rise in melatonin concentrations was absent. Furthermore, Fitzgerald and McManus [50] found that the administration of melatonin to mares failed to significantly alter the onset of the anovulatory period. The idea that melatonin reduces GnRH content in the hypothalamus is indirectly supported by Hart et al [51]. In their study, hypothalamic content of GnRH

varied within seasons and was lowest during mid-anestrus. However, different results were obtained by Silvia et al. [52], who observed no difference in the hypothalamic content of GnRH during different seasons. In a recent study in our laboratory, a marked reduction in secretion of LH occurring around the autumnal equinox and continuing throughout the winter anovulatory period did not appear to be accompanied by a similar reduction in pulsatile secretion of GnRH in the ICS [13]. This is somewhat paradoxical because, when GnRH is injected in a pulsatile manner into anovulatory mares during the spring at a rate as low as 2 μg/h, an increase in secretion of LH is observed coincidentally with follicular development and ovulation [6]. In the late winter (second week of January), pulsatile infusion of GnRH at a rate of 20 μg/h increased plasma concentrations of LH and caused ovulation within 12 d [10]. This suggests that GnRH is indeed limiting during the anovulatory period.

Seasonal reproduction in sheep is also influenced by day length and regulated by the pineal hormone, melatonin. However, contrary to the mare, melatonin has a positive effect on reproduction in the ewe [53]. The use of melatonin microimplants placed in the premammillary hypothalamic area (PMH) stimulated secretion of LH, suggesting that this is a melatonin target area mediating seasonal reproduction in the ewe [54]. However, exogenous treatment with melatonin that advances the onset of the breeding season in the ewe did not appear to do so by direct stimulation of LH and FSH concentrations in the peripheral blood [54]. To date, although there is some understanding of how melatonin influences reproduction in seasonal breeders, there remains a need for more work to precisely understand the mechanism involved in this process.

2.2.2 Gonadotropin-inhibiting hormone

Gonadotropin releasing hormone has historically been considered to be unusual among hypothalamic neuropeptides in that it appeared to have no direct antagonist, although some neurochemicals and peripheral hormones (opiates, GABA, gonadal steroids, inhibin) can modulate gonadotropin release to a degree [55]. Recently, several new neuropeptides involved in the regulation of GnRH have been found that have consequent effects on LH and FSH secretion. A novel neuropeptide involved in regulating reproduction is gonadotropin-inhibiting hormone (GnIH). This neuropeptide was found initially in seasonally-breeding avian species and is part of the so-called RF-amide-related peptide family [56]. Gonadotropin inhibiting hormone is a dodecapeptide with a C-terminal Leu-Pro-Leu-Arg-Phe-NH_2 sequence that has been shown to have negative regulatory effects of LH. Gonadotropin-inhibiting hormone-like immunoreactive neurons were localized throughout the encephalic and diencephalic regions in avian species; however, precursor mRNA for GnIH was found to be expressed only in the paraventricular nucleus (PVN), which indicates that the PVN may be the only site for synthesis of GnIH [57]. Similar gene sequences have been discovered in the human, rat and bovine hypothalamus encoding amino acid sequences very similar to GnIH, characterized as RF-amide related peptide-1 (RFRP-1), RF-amide related peptide-2 (RFRP-2) and RF-amide related peptide-3 (RFRP-3). This indicates that these three peptides may have evolved from a common ancestor [57,58,59].

In rats, intracerebral ventricular (ICV) injection of RFRP-3 reduced plasma concentrations of LH but did not alter plasma levels of FSH [58]. Bentley et al. [55] showed that there is a close proximity of GnIH fibers to GnRH fibers and neurons

throughout the hypothalamus in birds and mammals. It has also been demonstrated that GnIH decreases gonadotropin release from cultured anterior pituitary cells in songbirds and that GnIH-immunoreactive neurons inhabit larger areas in the hypothalamus at the end of the breeding season than at other times. Moreover, Ubuka and collaborators [60] demonstrated that melatonin induces the expression of GnIH in quails. Pinealectomy, combined with orbital enucleation, caused a concomitant decrease of GnIH precursor mRNA, content of mature GnIH peptide, and endogenous melatonin. Administration of melatonin to these birds caused a dose- dependent increase in expression of GnIH precursor mRNA and production of mature peptide. In addition, GnIH mRNA and GnIH peptide have been shown to be elevated during short days in quails, when melatonin secretion is known to be greatest [60].

In our laboratory, we have also observed GnIH neurons and fibers in close proximity to GnRH neurons and fibers in the hypothalamus of the mare. (unpublished data). These findings suggest that GnIH is present in the mare. It is possible that GnIH, perhaps controlled by melatonin, could contribute to the abrupt decline in secretion of LH in the mare in the fall and winter, thus serving as an alternative mechanism to explain the development of the winter anovulatory state [13]. However, additional research will be required in order to confirm this hypothesis and to determine where and how GnIH could work to effect such a result.

2.2.3 Kisspeptin

Kisspeptin is a neuropeptide produced in the hypothalamus that also belongs to the family of RF-amide proteins. Kisspeptin has an Arg-Phe-NH2 C-terminal sequence characteristic of this family of peptides that bind to the G-protein coupled receptor,

GPR54 and appears to act as a stimulator of GnRH secretion [61]. Messager and coworkers [61] showed that GPR54 transcripts colocalize with GnRH in neurons. They also demonstrated that the central administration of kisspeptin in sheep produces a dramatic release of GnRH into cerebrospinal fluid. Another recent study showed that constant intravenous infusion of kisspeptin into ewes induces a sustained release of LH and FSH during the breeding season [62]. During the anestrous season, intravenous treatment with kisspeptin at a rate of 12.4 nmol/h, for either 30 or 48 h, caused ovulation in 80% of treated ewes compared with 20% of saline-treated controls [62]. Dungan and collaborators [63] found that in mice the deletion of the GPR54 gene produces hypogonadotropic hypogonadism, without any other obvious phenotypic abnormalities. A different group of investigators showed that, in the rat, kisspeptin expression is up-regulated by estradiol at the level of the pituitary and that GPR54 is up-regulated by GnRH which exclusively targets gonadotrophs [64]. Administration of kisspeptide results in increase in FSH and LH secretion in the mare (ref attached), but its relationship to seasonality has not been investigated [65].

2.3 Control of seasonal breeding in mares

2.3.1 Artificial lighting

The universal birth date (January 1) for foals imposed by many breed organizations has caused a misalignment of the operational and natural breeding season of the mare. Supplemental lighting programs have been used for many years to induce earlier onset of spring transition in winter anovulatory mares. Exposure to 15-16 h of daylight, beginning in early to mid-December, stimulates follicular development and advances the time of ovulation in anovulatory mares [5]. Mares exposed to 24 h of light

continuously had increased circulating concentrations of LH in daily samples compared to mares exposed to 12 h of light and 12 h of darkness. Similarly, an increase in GnRH content of the hypothalamus in the 24-h light group was also observed [4]. Kooistra and Ginther [66] showed that 15.5 hours of light from the summer solstice through the following winter was an effective method for hastening the onset of the ovulatory season.

Scraba and Ginther [67] tested different lighting programs for their efficiency in hastening the onset of the ovulatory season. Fifteen and one-half h of light and 8.5 h of dark were applied starting at three different time of the year; summer (July, August), late-fall (November, December) and winter (January). All treated mares entered the ovulatory season earlier than control mares under natural day-length. However, the average date of the first ovulation after January 1 was one month earlier for the late-fall group than for either the summer or the winter group. Moreover, beginning the lighting program during the winter was less efficient than beginning the program in late-fall. A combination of artificial long-day photoperiod and 40 μg of the GnRH agonist, buserelin at 12 h intervals for 28 days during late winter/early spring increased serum concentrations of LH within 6 wk, whereas the mares in ambient lighting required 12 wk to respond to GnRH [68].

To investigate the influence of day length on the seasonal reproductive cycle of the stallion, Clay and Squires [69] assigned 21 stallions to one of three treatments: 1) control (natural photoperiod), 2) S-L (8 h light and 16 h dark for 20 weeks and then 16 h light and 8 h dark from December 2, 1982 until March 5, 1984), 3) S-S 8 h light and 16 h dark from July 16, 1982 until March 1984. The stimulatory effects of long photoperiod resulted in larger testes, decreased time to ejaculation and more sperm output for S-L stallions in February than November for both years of evaluation. However, total scrotal

width and sperm output for S-L stallions eventually declined, presumably as a consequence of photorefractoriness.

Artificial lighting programs are a good option for advancing the onset of cyclicity in mares. Unfortunately, they can often require a large investment in physical infrastructure.

2.3.2 Treatment of mares with exogenous GnRH

Hormonal treatment methods that have been used to accelerate the onset of cyclicity in mares have included the administration of both native GnRH and GnRH agonists. Hyland and collaborators [70] found that GnRH administered at a rate of 40-60 μg/h continuously for 28 days during the spring transitional period could induce fertile estrus. In studies conducted in our laboratory, continuous administration of low-dose GnRH (2.5 to 5 μg/h) was successful in stimulating a small increase in LH and hastening the onset of first ovulation in mares exhibiting persistent anovulation during the ovulatory season [11]. Becker and Johnson [6] treated seasonally anovulatory mares and cyclic mares with GnRH in both a pulsatile (20 μg/h) and continuous manner (2-20 μg/h). They concluded that the mode of GnRH infusion (i.e., pulsatile vs continuous) does affect differentially the secretion of LH. Anovulatory mares that were treated with pulsatile GnRH ovulated on average 2.8 d earlier than those treated with GnRH continuously, although all mares ovulated in the three groups. Moreover, circulating concentrations of LH in cyclic mares treated with a constant infusion of GnRH were lower on the day of ovulation than in both control mares and those treated with GnRH in a pulsatile manner. McCue and collaborators [7] showed that seasonally anestrous mares treated with native GnRH (10 μg/h) injected subcutaneously with a peristaltic pump in a pulsatile manner

resulted in a greater follicular development than that in mares treated with 10 μg of a GnRH agonist (buserelin) injected s.c twice daily. Concentrations of serum LH in mares induced to ovulate with native GnRH were greater (7.4 ng/mL) than those induced with the agonist (1.8 ng/mL). Both treatments induced ovulation in 11/14 and 10/17 mares for the native GnRH and analog, respectively, and each ovulation was followed by a normal luteal phase.

Although a number of studies have shown that the onset of follicular development and ovulation in anovulatory mares can be advanced with GnRH treatment beginning soon after the winter solstice, there is only one reported study in which this has been attempted before the winter solstice. Collins et al. [12] showed that continuous, low-dose infusion of GnRH (2.5 μg/h-5.0 μg/h), beginning in October and continuing through March, was unable to prevent the onset of seasonal anovulation in the fall or to advance the timing of ovulation in the spring. Similar doses of GnRH were previously shown to be effective in about 80% of persistently anovulatory mares during the natural breeding season. One explanation for the different responses noted between the two studies is that gonadotropes may be less sensitive to GnRH stimulation before compared to after the winter solstice. Day length decreases slowly before the winter solstice and increases slowly thereafter. A previous report indicated that photoperiod and estradiol interact to increase the synthesis and secretion of LH in anestrus mares [71]. However, untreated mares under natural lighting have very little releasable LH in the pituitary even as late as March 31 [12]. Therefore, endogenous stimulation of gonadotropes by GnRH under natural photoperiod should not substantially complicate the ability to compare responsiveness to GnRH in December vs February.

The sustained release of GnRH agonist from implants has also been shown to hasten ovulation. Treatment with deslorelin, a GnRH analog, is a consistent method of inducing ovulation in cyclic mares during the breeding season [72] and is currently marketed in a sustained-release implant form as Ovuplant (Ft. Dodge Animal Health, Overland Park, KS). Squires and collaborators [72] conducted a series of dose-response studies to evaluate the efficacy of this synthetic agonist for hastening ovulation of mature preovulatory follicles in nonlactating cyclic mares. They determined that 2.2 mg/mare of deslorelin induced ovulation in most of the mares having follicles of at least 37.7 mm diameter within 48 hours. There was no effect of GnRH on pregnancy rates. On the other hand, Mumford et al. [73] reported that use of a GnRH agonist in implant form, administered at varying doses to induce ovulation in transitional mares, resulted in only 15 of 100 mares ovulating within 30 days of treatment. Similarly, Fitzgerald et al. [74] found that constant administration of the GnRH agonist, goserelin acetate, failed to induce ovulation effectively in anovulatory mares.

Harrison et al [68] treated transitional mares for 28 days with a GnRH agonist (buserelin), administering 40 ug of buserelin per injection every 12 hours i.m.. He reported ovulation in 7/15 treated mares compare to 0/15 ovulations in the untreated control group.

2.3.3 Desensitization of the anterior pituitary to GnRH

In mammals, continuous treatment with GnRH generally results in GnRH receptor down-regulation, producing a desensitization of anterior pituitary gonadotropes to GnRH. In mares, this down-regulation seems relatively less robust. Porter and Sharp [9] studied the ability of mares and sheep to respond to continuous treatment with GnRH

during diestrus. Results indicated that GnRH administered continuously to ewes at a rate of 20 µg/h resulted in an initial increase followed by a decline to basal concentrations that are typical of GnRH receptor down-regulation. However, treatment of mares continuously with GnRH up to 110µg/h resulted in sustained secretion of LH. In the same study, mares and ewes were treated with GnRH using both pulsatile and continuous modes of treatment. Doses used for the mares were 25 µg/h for hourly- pulsatile and 110 µg/h for continuous treatments, respectively. Doses used for the ewes were 25 ng per hourly pulse and 2.5 µg/h continuously for 5 days. For both treatments, there was an increase in LH at day 5 in mares, while in ewes continuous treatment produced a marked suppression in secretion of LH. Concentrations of LH in ewes treated with pulsatile GnRH did not differ from contols [9]. In order to understand this phenomenon, an additional study was completed to investigate the structure and intracellular trafficking of the equine GnRH receptor [75]. Results indicated a slow rate of receptor-mediated endocytosis which apparently contributes to the observation that equine pituitary cells maintain the ability to bind radiolabeled GnRH in the presence of excess unlabeled GnRH. Additionally, there are a few amino acid substitutions that appear distinct to the equine GnRH receptor gene sequence. These may contribute to the relative capability of the equine receptor to resist desensitization [75]. However, the basis of the ability of continuous GnRH administration to stimulate secretion of LH in the mare remains to be fully elucidated. One early observation made by Alexander and Irvine [3] was that when the exogenous GnRH signal is continuous in the mare, LH secretion seems to remain pulsatile in pituitary venous blood. However, an in-depth study to test this hypothesis using the ICS cannulation technique remains to be conducted. If confirmed, then the

continued pulsatility of LH release would suggest that small endogenous GnRH pulses are being superimposed on the continuous background of exogenous GnRH. This argues that during continuous infusion the GnRH signal to the pituitary remains, in part, intermittent [3].

The continuous administration of potent GnRH agonists in the mare results in findings different from those summarized above for native GnRH. Use of the GnRH analogue, deslorelin acetate, in cyclic mares to induce ovulation prolonged the interovulatory interval by 6.2 days when luteolysis was induced with PGF2α. These mares had suppressed LH and FSH concentrations for up to 11 days after ovulation [76]. When a group of geldings treated with progesterone and estradiol to mimic the estrous cycle of the mare were treated with deslorelin acetate (Ovuplant), the implant suppressed both LH and FSH concentrations for at least 14 days. Similar observations were made for a group of stallions [77]. Importantly, prolonged exposure of mares to residual hormone remaining in the Ovuplant implant resulted in ovarian quiescence for an extended period up to 17.5 ± 7.2 days in some mares. Farquhar and collaborators [78] found that removal of the deslorelin implant in mares after 2 days of treatment prevented the subsequent decrease in gonadotropin concentrations and follicular growth.

In male sheep [79], dogs [80,81], rhesus monkeys [82,83], rats [84,85] and humans [86], continuous exposure to GnRH agonists suppresses the activity of the pituitary-gonadal axis. In a study with postpartum acyclic cows, while continuous long-term GnRH and GnRH-agonist treatment induced an earlier first ovulation, it resulted in failure of a second ovulation following luteal regression [87]. D'Occhio et al. [88] used 200 or 400 ng of native GnRH/kg body weight per h and 5.5 or 11 ng of the GnRH

buserelin/kg body weight per h that were delivered continuously using osmotic mini-pumps designed to remain active for 28 days. All treatments increased mean basal circulating LH concentrations, but 3 cows, one each from the 400 ng GnRH, 5.5 ng buserelin and 11 ng buserelin , showed evidence of low levels of LH and FSH by failing to show a second ovulation. Authors explained that this could have been due to insufficient FSH stimulation for early follicular development, or the absence of an endogenously driven LH surge [88]. A further study was conducted to examine if buserelin could be used as a controlled, reversible suppressor of the estrous cycle in beef heifers and cows [89]. Two groups of cyclic heifers were treated with either two or four buserelin implants (3 mg) and degree of cessation of estrous cycles was monitored. Both groups stopped cyclicity as indicated by basal progesterone concentrations (0.2 ng/ml). When cows were implanted with four implants on day 7 of their ensuing cycle for either 28 or 56 days, those cows ceased exhibiting ovarian cycles as well; however, when implants were removed, estrous cycles resumed in all animals [89]. A different group of investigators treated prepubertal heifers with GnRH subcutaneously at a rate of 5 µg/h for 12 days. All heifers showed an immediate pituitary response to GnRH stimulation, with serum values of LH reaching preovulatory surge levels within 2-4 h after pump insertion that remained higher than pretreatment values until treatment removal. They concluded that continuous infusion of the GnRH at a rate of 5 µg/h for 12 days seems to be effective in stimulating a long-lasting pituitary response in prepubertal heifers [90]. Similarly, when prepubertal bulls were treated with a GnRH agonist, azagly-nafarelin, for an extended period, basal concentrations of LH were greater for the treated group than they were for controls. However, frequency of LH pulses in the agonist-treated group was

reduced to less than one pulse in 24 h [91]. D'Occhio and Aspden [92] treated young bulls with deslorelin using dosages ranging from approximately 0.15 to 0.29 µg deslorelin/kg live body weight per day. All dosages increased plasma LH concentrations that declined after 24 h; however LH pulses were abolished by the treatments. The data suggested that the anterior pituitary in bulls became desensitized after receiving LHRH agonist treatments. Collectively, the foregoing suggests that basal concentrations of LH can be chronically elevated to a minor degree in some species using continuous GnRH and GnRH agonist treatments. However, these effects appear to be working through signaling mechanisms within the gonadotrope that somehow elevate LH at the baseline while abolishing typical pulsatile release patterns associated with normal anterior pituitary and gonadal function.

CHAPTER III

EFFECTS OF CONTINOUS TREATMENT WITH EXOGENOUS GnRH ON TEMPORAL CONCENTRATION PATTERNS OF LH IN THE INTERCAVERNOUS SINUS OF THE CYCLIC AND ANOVULATORY MARE

3.1 Introduction

The continuous administration of exogenous GnRH and its synthetic agonists to most mammalian species results in a general down-regulation of the GnRH receptor, thus desensitizing the anterior pituitary to their stimulatory effects and markedly reducing secretion of LH. However, in the horse, continuous infusion of native GnRH at doses greater than 100 µg/h remains stimulatory [9], although this is not generally true for its more potent synthetic agonists [78]. In addition, Alexander and Irvine [3] proposed that secretion of LH in the mare remains pulsatile even when GnRH stimulation is constant. This hypothesis has not been critically tested, and little is known about the hypothalamic-pituitary interrelationships that would subserve such a unique phenomenon.

Mares also exhibit profound reproductive seasonality, and varying strategies have been used to accelerate gonadotrope recrudescence for secretion of breeding season levels of LH [6,7,10]. This, in turn, would promote earlier breeding and subsequent foaling during the calendar year. Previous work in our laboratory has shown that low doses of continuously-administered, native GnRH (2.5-5 µg/h sc) are unable to prevent the development of or to reduce the length of winter anovulation [12]. However, the administration of similar doses in April successfully induced follicular development and ovulation in approximately 80 % of persistently anovulatory mares, suggesting that the

anterior pituitary may be less responsive before or near the winter solstice than after [11]. Therefore, the question remains as to whether the seasonal anovulatory state is controlled solely by a limitation of endogenous GnRH secretion or changes associated with pituitary responsiveness. Additional studies in our laboratory, utilizing the intercavernous sinus (ICS) sampling technique [13] that allows the measurement of pulsatile secretion of both GnRH and gonadotropins, found that nor frequency nor amplitude of GnRH pulses changed over season, resulting in the conclusion that GnRH release may not be limiting during the anovulatory season [13]. These findings are contrary to accepted dogma which suggests that the lack of an adequate GnRH signal to the anterior pituitary is the primary cause of seasonal anovulation. Therefore, additional experiments are required in order to resolve this conflict and to more fully understand the unique interrelationships that appear to exist between the hypothalamus and anterior pituitary in horses. The objectives of the current studies were 1) to investigate the responsiveness of gonadotropes to continuous GnRH infusion in cyclic mares during the breeding season, and 2) to examine whether discernable changes in anterior pituitary responsiveness to continuous GnRH exists in anovulatory mares near and after the winter solstice.

3.2 Materials and methods

The Institutional Agricultural Animal Care and Use Committee of the Texas A&M University approved in advance all procedures used in these studies.

3.2.1 Experiment 1

3.2.1.1 Animals

Ten Quarter horse mares, provided by a local breeder were maintained on pasture (Coastal Bermuda grass) at the Texas AgriLife Research Station-Beeville and

supplemented with a mixed grain concentrate (14% crude protein; Falls City Milling, Falls City, TX) to maintain a minimum body condition of 5 (1 to 9 scale). Eight of the ten mares had suckling foals at side until weaning at 4 mo of age. Estrous cycles were monitored by daily teasing with two stallions and transrectal ultrasonography was employed three times weekly to estimate day of ovulation (define as Day 0). Teasing scores used were as follows: 1-breaks down, urinates; 2-winking, intense interest in stallion; 3-some interest in stallion; 4-passive; 5-rejects stallion.

3.2.1.2 Experimental procedures

Mares were assigned randomly to one of two groups: 1) GnRH (continuous intravenous infusion of 100 µg/h; n=5) or 2) Control (continuous intravenous infusion of saline; n=5). The GnRH or saline treatments were delivered via an Alzet osmotic pump (Model 2ML1; Alza Scientific Products, Palo Alto, CA) that was placed surgically under the skin of each mare and connected to a catheter placed in the external jugular vein. Pumps were inserted between Days 3 and 6 after ovulation (Day 0 of treatment). Pumping rate for the lot of pumps used in this experiment was 9.15 µl/h, allowing for continuous function up to 7 d. Therefore, concentration of GnRH in the loaded pumps was 10.9 µg/µl, resulting in delivery of GnRH at a rate of 100 µg/h. Daily blood samples from the contralateral jugular vein were collected by venipuncture beginning immediately before pump insertion and continued until pump removal on Day 6 of treatment. On Day 3 after initiation of treatments, (Days 6 to 9 after ovulation), the ICS was catheterized via the facial vein for intensive blood sampling [45]. Catheterization was performed under light sedation and using a local anesthetic as described in detail below. Catheterization of ICS was used for collection of intensive blood samples on Days 4 and 6 of the

experiment. On each day of sampling, mares were heparinized (30,000 IU sodium heparin, iv.), tied loosely and provided with hay and water during the procedure. On the day following ICS catheterization (Day 4 after onset of treatment; Day 7 to 10 after ovulation), 5-mL ICS blood samples were collected over 15 sec every 5 min during 8 h to determine concentrations of LH during the luteal phase by radioimmunoassay (RIA). Samples were placed into tubes containing 50 µl of a 5% EDTA-heparin solution (10,000 IU/mL) to prevent coagulation and 100 µl of a 50 mM solution of Bacitracin (Sigma Chemical Co., St Louis, MO USA). The latter was added to minimize metabolism of GnRH. However, for the purpose of this report, GnRH was not measured in these samples. Samples were placed immediately on ice and centrifuged (5125 x g) for collection of plasma within one h of collection. Plasma was stored at -20 ^{o}C until used for hormone analysis. Immediately following the first intensive ICS blood sampling, mares were injected with 2 mL of $PGF_{2\alpha}$ im (10 mg Lutalyse, Pfizer, New York, NY) to regress the CL. A second intensive ICS blood sampling was performed 36 hours after $PGF_2\alpha$ injection (Day 6 of experiment, early follicular phase; Fig 1). Upon completion of the last ICS sampling period, catheters and pumps were removed and mares were returned to the pasture. The skin overlying the site of cannulation and pump was inspected daily and treated with a topical antiseptic/antibiotic as required until healed. A time-line of the experimental procedures is shown in Fig. 1.

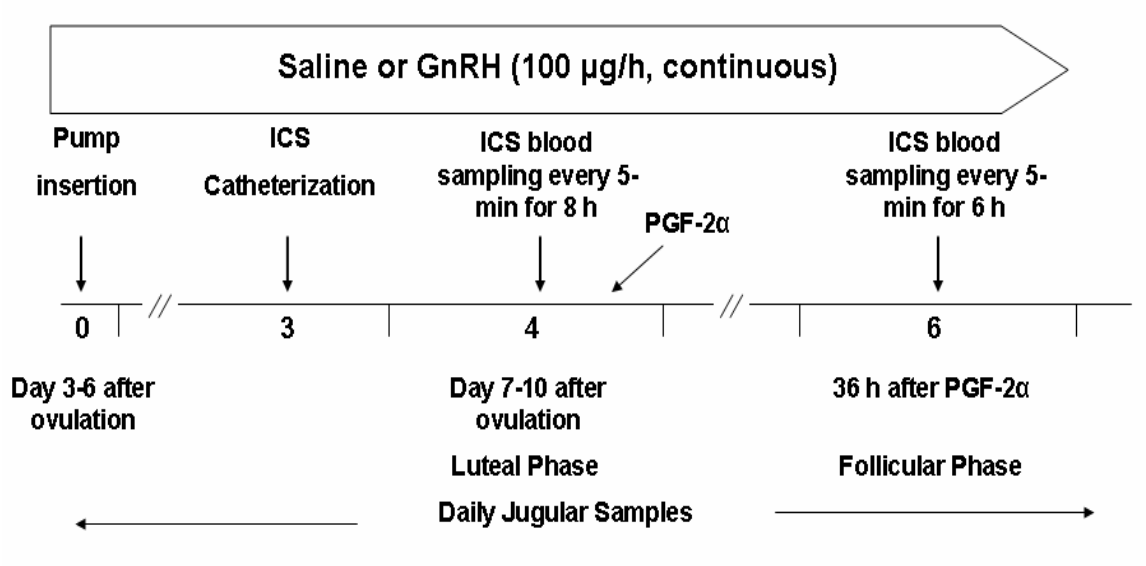

Figure 1**.** Time line of procedures for experiment 1 relative to day of ovulation and day of experiment in individual mares.

3.2.1.3 Intercavernous sinus catheterization procedure

Catheterization of the intercavernous sinus (ICS) was performed following procedures described previously [3,13,46]. The mare was placed in a stock and sedated with detomidine (Dormosedan, 20-40 µg/kg BW Pfizer, Ney York, NY). The facial vein lies parallel to and along the anterior border of the mandible. An area centered over the line of the mandible and extending in all directions for approximately 2 to 2.5 cm was clipped, scrubbed and disinfected for aseptic surgery. The facial vein was detected by palpation and an area (approximately 1 cm x 1 cm) overlying and surrounding the vein, but below the facial crest, was infiltrated subcutaneously with 2% Lidocaine HCl (Vedco, Inc, St Joseph, MO). A 1-cm skin incision was made over the vein, and the vein was exteriorized using blunt dissection and held in the exteriorized position by placing a sterile probe between the vein and the underlying tissue. A small cut was made in the vein while occluding blood flow, and a Tygon catheter (0.04 i.d. x 0.07 o.d.; Norton

Performance Plastics Co, Akron, OH) was inserted into the vein. A flexible stylette was maintained in the catheter and the catheter was gently threaded up the vein. The objective was to thread the catheter cranially to the base of the cranial cavity. A lateral radiograph was taken to visualize the position of the catheter and verify that it was located in the ICS (Fig 2). If the catheter was off target, it was removed and rethreaded. Once in place, the stylette was removed and a heparin (10,000 IU/mL) lock was placed in the tubing to prevent clotting. The tubing was sutured with synthetic sutures (Supramid; Burns Veterinary Supply, Inc, Westbury, NY) above and below its exit to the skin and the skin was partially closed and dressed with a wound dressing. During ICS blood sampling, the mares were heparinized with 30,000 IU heparin every 3 h. Catheters, when not being used for intensive sampling, were flushed once or twice daily with 3 to 5 mL heparinized saline (10,000 IU).

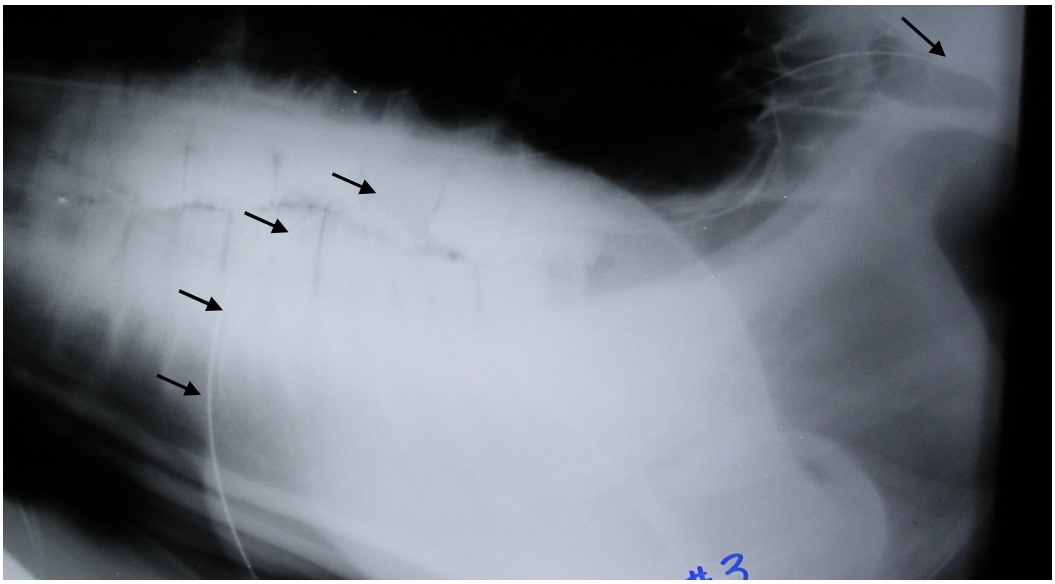

Figure 2**.** Lateral radiograph post-catheterization. Arrows help identify the catheter with the guide wire still in place as it travels toward the cranium into the ICS and the tip resting just below the orbit of the eye.

3.2.1.4 Alzet osmotic mini-pump insertion

The mare was placed in a stock and sedated with detomidine (Dormosedan, 20-40 µg/kg BW) if required. An area on the jugular grove measuring approximately 3 x 3 cm was clipped, scrubbed with an iodophore, and disinfected with dilute chlorhexidine solution (Vedco, Inc, St Joseph, MO) and povidone iodine solution (Prepodyne, WestAgro, Kansas, MO). Three to five ml of 2% Lidocaine HCl was injected sc and a 1.5 to 2-cm incision was made through the skin with a sterile scalpel. A sterile, blunt instrument was used to expand the subcutaneous space making a pocket to accommodate the Alzet pump containing GnRH diluted in saline or saline only. Another small incision was made for inserting a 12-gauge needle in the jugular vein. A polyethylene catheter (Becton Dickinson and Company, Sparks, MD) was inserted in the vein trough the needle and connected subcutaneously to the pump. The incision was closed with #2 synthetic sutures (Supramid) or other suitable non-absorbable suture that is left in place for 7 days. The wound was dressed with a wound dressing. On the last day of the experiment (Day eleven), the incision was re-opened, the pump was removed, and the incision was closed with a non-absorbable suture. Sutures were removed 7 d later.

3.2.2 Experiment 2

3.2.2.1 Animals

Fifteen mares were maintained on pasture (Coastal Bermuda grass) and supplemented with a grain mixture concentrate as for Experiment 1 to maintain mares in good body condition. Before the study, all mares were examined weekly using transrectal ultrasonography to determine onset of the seasonal anovulatory (non-cycling) period. In addition, blood samples from the jugular vein were collected twice weekly for

determination of serum concentrations of progesterone as an adjunct to ultrasonography for assessing luteal function..

3.2.2.2 Experimental procedures

Mares were classified as anovulatory based on serum concentrations of progesterone (P4) less than 1 ng/ml for at least 3 wk, visual confirmation of absence of corpora lutea by transrectal ultrasonography and no follicles greater than 30 mm. Three mares were considered to have residual follicular activity because they had follicles larger than 30 mm, however they had not ovulated in a 21 days. At the onset of the experiment (approximately Dec. 5), mares were assigned randomly to a control or one of two GnRH treatment groups: GnRH-20, 20 µg/h; and GnRH-100; 100 µg/h. A sham pump constructed of Silastic tubing (Dow Corning Corporation, Midland, MI) and filled with medical grade Silastic adhesive (Dow Corning Corporation, Midland, MI) was surgically implanted under the skin for 14 days in control animals. Alzet osmotic minipumps (model 2ML2; Alza Scientific Products, Palo Alto, CA) were surgically implanted under the skin to deliver GnRH treatments for 14 days. A blood sample was collected daily for 14 days from the jugular vein to determine serum concentrations of LH. Treatments were applied between December 5 and 20. Transrectal ultrasonography was performed every 2 to 3 days to monitor ovarian follicular dynamics. At the end of the 14-day experimental period, all pumps and sham pumps were removed. The experiment was repeated beginning on February 15 with the same mares assigned to the same groups.

3.2.2.3 Alzet osmotic mini-pump insertion

The procedure for pump insertion was identical to that described for Experiment 1 except that hormone delivery was subcutaneous and pumps were loaded with GnRH in saline at a concentration of 4.27 μg/μl and 21.36 μg/μl of GnRH for delivery of the 20 μg/h and the 100 μg/h doses, respectively. Pumping rate of the lot of pumps used in this experiment was 4.6 μg/h.

3.2.3 Hormone analyses

Concentrations of LH were analyzed by double antibody RIA for all intensive samples collected, as validated previously in this laboratory [11]. A highly-purified equine LH (eLH AFP-5130A) preparation was used for both iodinated tracer and standard. An anti-eLH antiserum (AFP-240580) is used at a dilution of 1:120,000 which yielded 35.9% binding on average at B/O. The sensitivity, intra- and interassay coefficients of variation (CV) for experiment 1 were 0.1 ng/ml, 5%, and 4.1% respectively. For experiment 2, the sensitivity, intra- and interassay CV were 0.1 ng/ml 13.8% and 1.4% respectively. Concentrations of progesterone in serum were measured using a commercial single antibody kit (Diagnostic Products Corporation, Los Angeles, CA) for all jugular samples collected. All samples within each experiment were analyzed for progesterone in a single assay. Sensitivity for progesterone assays was 0.1 ng/ml, and intra-CV was less than 2% for Experiments 1 and 2.

3.2.4 Pulse analysis

As noted earlier by Irvine and Alexander [45], patterns of episodic secretion of LH determined in the ICS often do not conform to those commonly described in the peripheral blood. Furthermore, existing pulse detection algorithms commonly used for

detection of pulses of hormones did not provide a consistent assessment of apparent high frequency episodes of LH observed in Experiment 1. Therefore, LH data for intensive sampling in Experiment 1 were transformed using 3-point rolling average to reduce background noise and assist in the identification of episodes of LH release. This process improved the detection of secretory episodes and confirmed episodes identified subjectively. A secretory episode was defined as an elevation of LH from the baseline of at least 0.1 ng/ml that was sustained during two consecutive samples. Therefore, this approach was utilized for final pulse detection analyses for LH. Duration of secretory episodes was determined as the time elapsed from the first elevation from the baseline to the following nadir

3.2.5 Statistical analysis

Data were analyzed using the Proc Mix procedure of SAS (SAS Inst., Inc., Cary, NC). For experiment 1, the statistical model tested the effect of treatment on frequency and duration of episodes, and mean concentrations of LH, using "phase" as the repeated variable and "mare(treatment)" as the subject. To test the effects of treatment on daily concentrations of LH and P4, "mare(treatment)" was used as the subject, "treatment" used as source of variation and "day' and separately "phase" as the repeated variables.

For experiment 2, the statistical model for LH concentrations and changes in follicular size included main effects of treatment with season used as the repeated variable and mare as the subject. Because there was a significant treatment by season interaction, day was used as the repeated variable to test the effects of "treatment" within each season for LH concentrations analysis. There was no season interaction for changes in follicular size therefore data from both seasons were pooled together to proceed with

the analysis. The Catmod procedure was used to analyze frequency of ovulation and frequency of estrus.

3.3 Results

3.3.1 Experiment 1

Figure 3 represents a comparison of hormone data plotted for Mare 8 using values from 5-min samples and those generated using a 3-point rolling average. Figure 4 represents least-squares mean concentrations of LH during luteal (Day 4 post onset of treatment) and follicular (Day 6, following prostaglandin administration) phases, respectively, for control and GnRH-treated groups. Concentrations of LH were markedly greater for the GnRH-treated mares compared to controls; and within the GnRH treated mares, concentrations of LH were greater during the luteal phase than during the follicular phase ($P < 0.0001$)

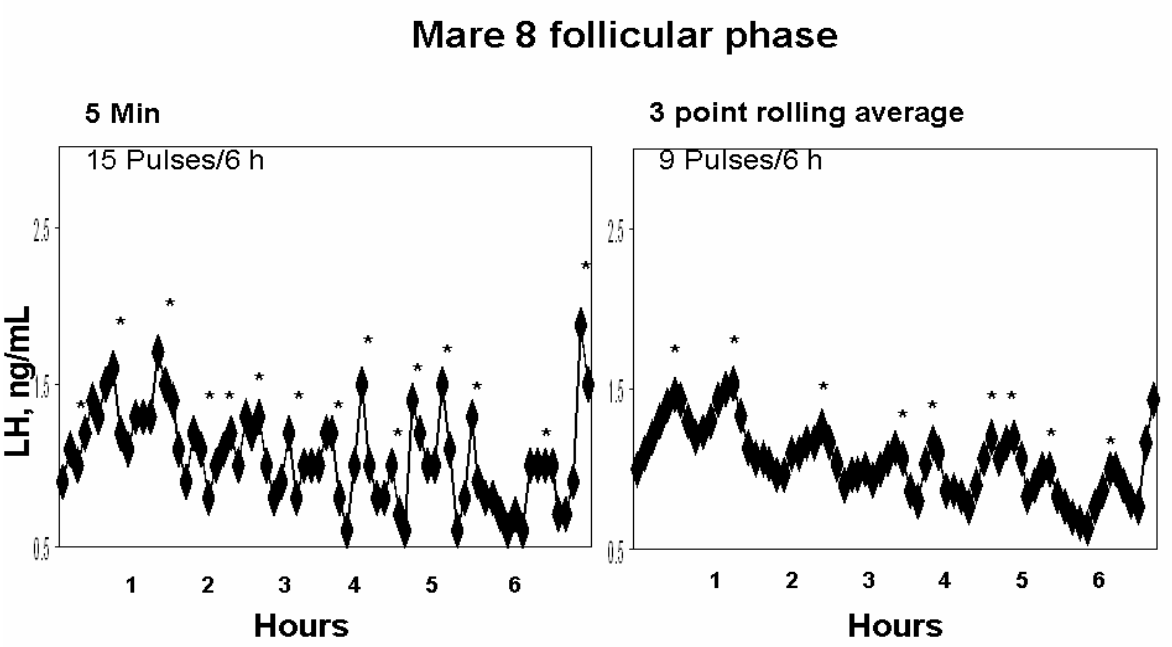

Figure 3. Five-min (left panel) and transformed three-point rolling average (right panel) patterns of LH in one representative control mare (Mare 8) during the follicular phase.

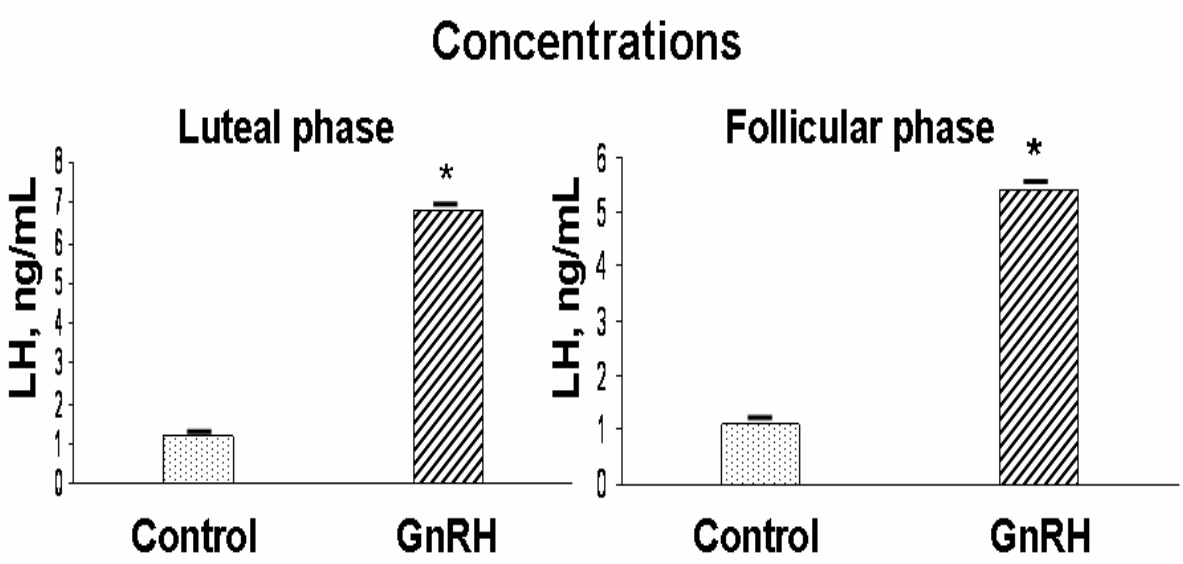

Figure 4. Least-squares mean concentrations of LH (ng/ml ± SEM) during luteal and follicular phases determined in ICS blood samples collected at five-min intervals in five control and five GnRH-treated mares. *Means differed ($P < 0.01$).

Table 1 summarizes the number and duration of LH secretory episodes during both luteal and follicular phases in control and GnRH-treated mares. During the luteal phase of control mares, the identified long-lasting episodes of LH release were followed by one to three secondary episodes. Treatment with GnRH disrupted the infrequent, large secretory episodes of LH release and markedly increased ($P < 0.01$) the mean baseline and frequency of episodes during the luteal phase (Fig. 5). Duration of episodes during the luteal phase lasted longer for the control group ($P< 0.001$) than they did for the GnRH treated group. During the follicular phase, a high-frequency, episodic pattern of LH secretion characteristic of this phase was observed in control mares. Treatment with GnRH increased ($P < 0.001$) the number of secretory episodes during the follicular phase (Fig. 5). Duration of episodes of LH release lasted longer for the control group than it did for the GnRH treated animals ($P< 0.001$).

Table 1. Least-squares mean (± SEM) number and duration of LH secretory episodes during luteal and follicular phases.

Treatment	Number of episodes (per h)		Duration of episodes (min)	
	Luteal phase	Follicular phase	Luteal phase	Follicular phase
Control	0.175 ± 0.2a	0.9 ± 0.2a	91.2 ± 6.1a	43.5 ± 2.2a
GnRH	1.1 ± 0.5b	1.3 ± 0.4b	37.8 ± 2.0b	34.7 ± 2.2b

*Means differ between treatments ($P < 0.001$).

Mean daily peripheral concentrations of LH are summarized on figure 6. Mean LH did not differ on day 0, but 24 h after GnRH administration concentrations of LH increased 2.8 fold ($P<0.04$) and remained elevated throughout the experiment compared to controls. Overall mean peripheral concentration of LH during the experimental period where greater for the GnRH treated mares compared to controls ($P < 0.001$) (Fig. 6). There was no treatment by phase interaction; therefore data were pooled by treatment. GnRH treatment also increased ($P<0.03$) mean serum progesterone concentrations 1.5 fold during the luteal phase (experimental Days 0 to 4, Fig. 7). Prostaglandin administration on Day 4 of the experiment regressed the CL in all mares, although mean progesterone concentrations were higher for the GnRH treated mares (0.7 ng/ml) than control mares (0.2 ng/ml) on the day of the second intensive sampling (follicular phase).

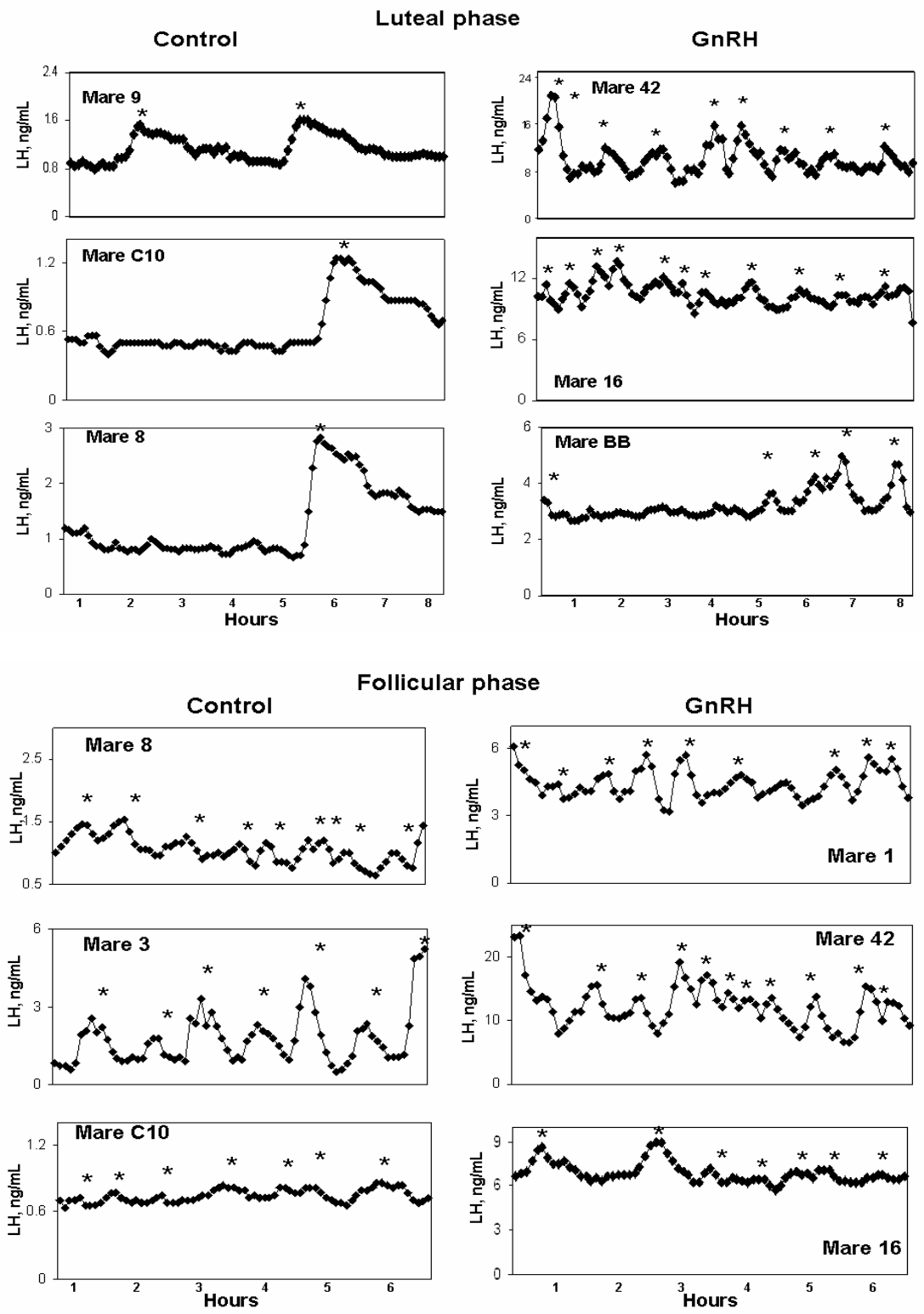

Figure 5. Patterns of LH secretion (three-point rolling averages) in three representative control and three GnRH-treated mares during follicular (top panel) and luteal phases (bottom panel) respectively. Pulses are denoted by an asterisk.

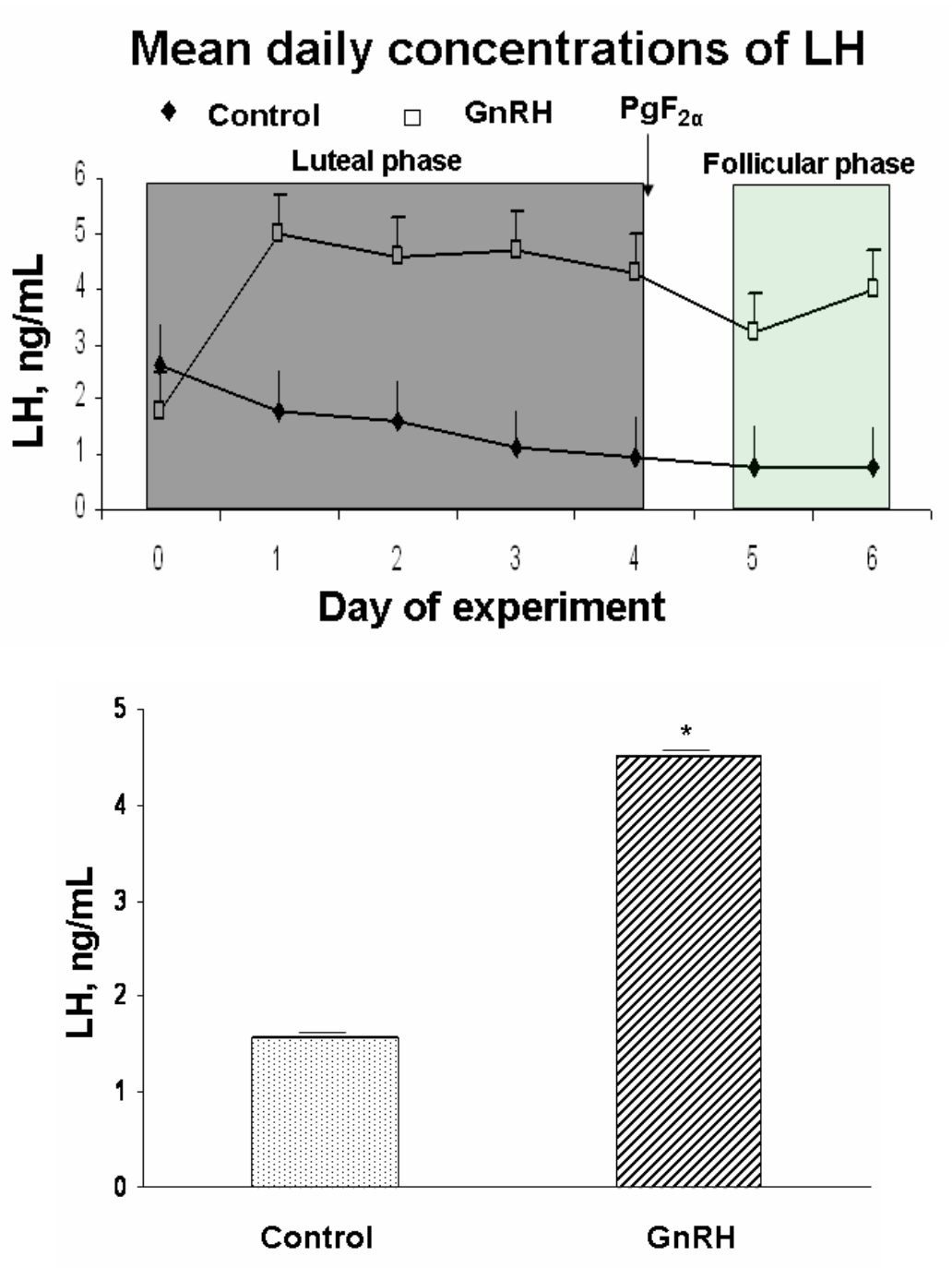

Figure 6. Mean daily concentrations of LH (ng/ml ± SEM) throughout the experimental period (top panel) and overall mean concentrations of LH (ng/ml ± SEM) (bottom panel). Means did not differed on Day 0 but they did from Day 1 through Day 6 ($P < 0.04$) (top panel) and means differed between groups ($P < 0.001$) (bottom panel).

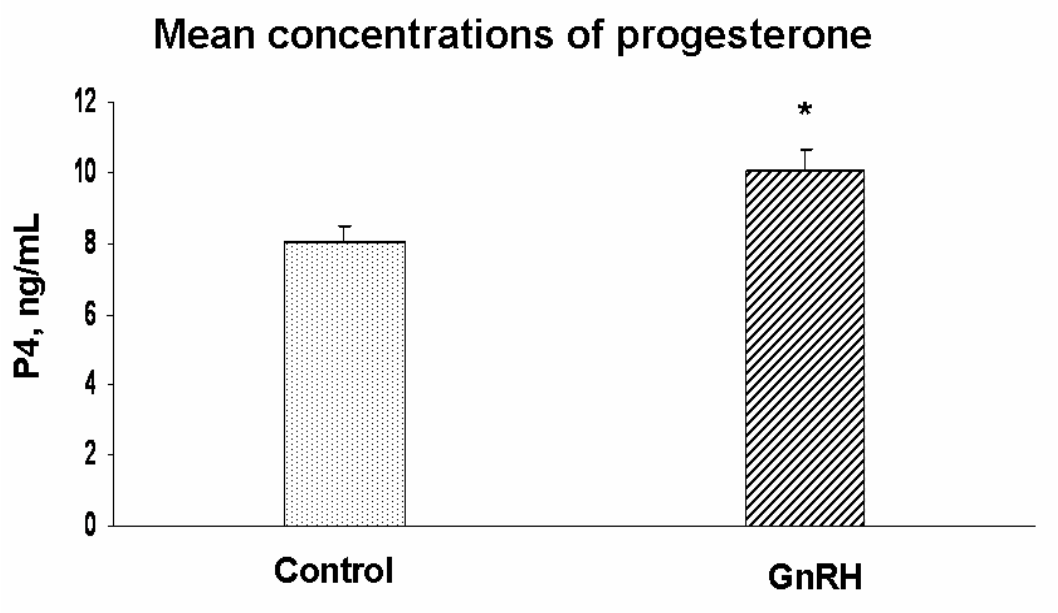

Figure 7**.** Mean serum concentrations of progesterone (± SEM) during the luteal phase. *Means differed between groups. (P< 0.03).

3.3.2 Experiment 2

The three mares with residual follicular activity were each assigned randomly one to each group, Control, GnRH-20 and GnRH-100. All mares were anovulatory when the experiment was repeated in February.

3.3.2.1 Hormonal responses

Near the winter solstice, mean concentrations of LH increased beginning one day after onset of GnRH treatments in both GnRH-20 and GnRH-100 treatment groups, continued to increase through Days 11 to 14 (Fig. 8), and were markedly greater overall than controls (Fig. 9). Mean concentrations of LH increased 10-fold in the GnRH-100 group compared to controls, but only 2.4-fold in the GnRH-20 group ($P < 0.001$).

When the experiment was repeated nearly 2 mo after the winter solstice, an increase in mean concentrations of LH was noted only in response to GnRH-100 beginning one day after treatment onset. Mean concentrations of LH increased 4.6-fold

in the GnRH-100 group but no changes were measured for the GnRH-20 compared to controls (Fig. 10). The GnRH-20 treatment did not produce changes in LH concentrations throughout the second season compared to controls (Fig. 10). Overall, mean concentrations of LH were greater ($P < 0.001$) in GnRH-100 than in controls and GnRH-20 groups (Fig. 11).

3.3.2.2 Ovarian morphology

Season did not have an effect on follicle size changes between the two seasons. Therefore, follicle data were pooled across seasons. Mean follicle size did not differ among groups at the beginning of each experiment (Day 0; Fig. 12), But mean largest follicle for both GnRH-20 and GnR-100 groups was larger (26.6 and 31.2 mm respectively) than the mean largest follicle for controls (19.1 mm) during the experimental period (Fig. 13). However, when changes in follicle size, determined as the difference between mean size of largest follicle and mean size of follicle on day 0 of the experiment, only in the GnRH-100 group had changes in follicular size ($P < 0.001$) (Fig. 14).

3.3.2.3 Frequency of ovulation

Frequency of ovulation was not affected by season and a higher proportion of mares in the GnRH-100 group than in the other groups ovulated during both seasons compared to the other groups ($P < 0.007$). Four of 5 mares treated with GnRH-100 ovulated during the first season (winter solstice) and 2 of 5 mares in this group ovulated during the second season (two months post-winter solstice). One mare in the 20-µg/h group ovulated during the first season (winter solstice); this was the mare observed with residual follicular activity in this group. No ovulations occurred during the second

season for the GnRH-20 group and no ovulations occurred in the control group during either of the seasons (Table 2).

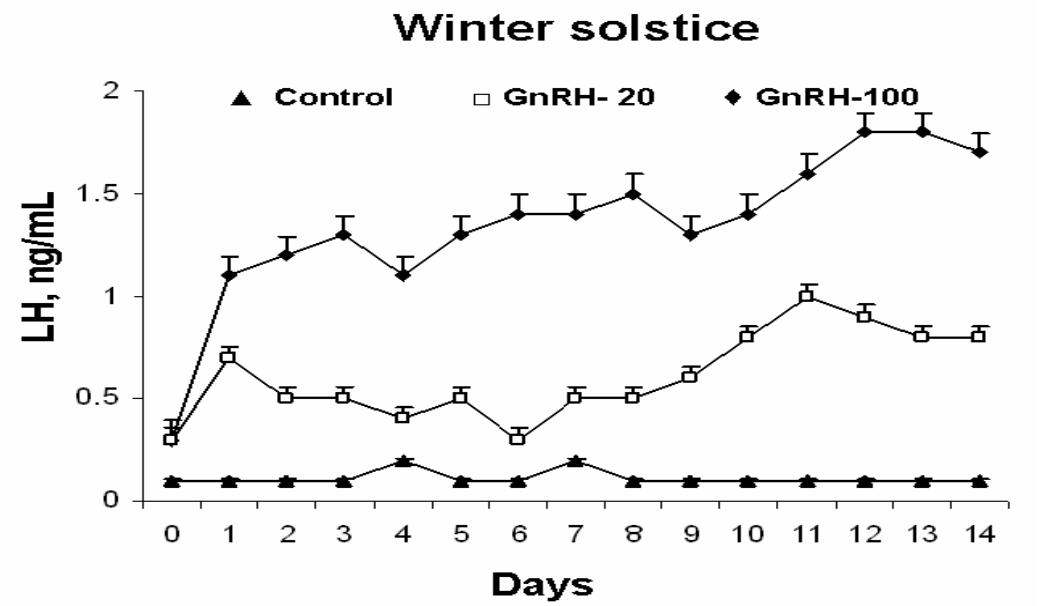

Figure 8. Daily mean serum concentrations of LH (ng/ml ± SEM) in control mares and mares in the GnRH-20 and GnRH-100 groups near the winter solstice (December 5 through 20).

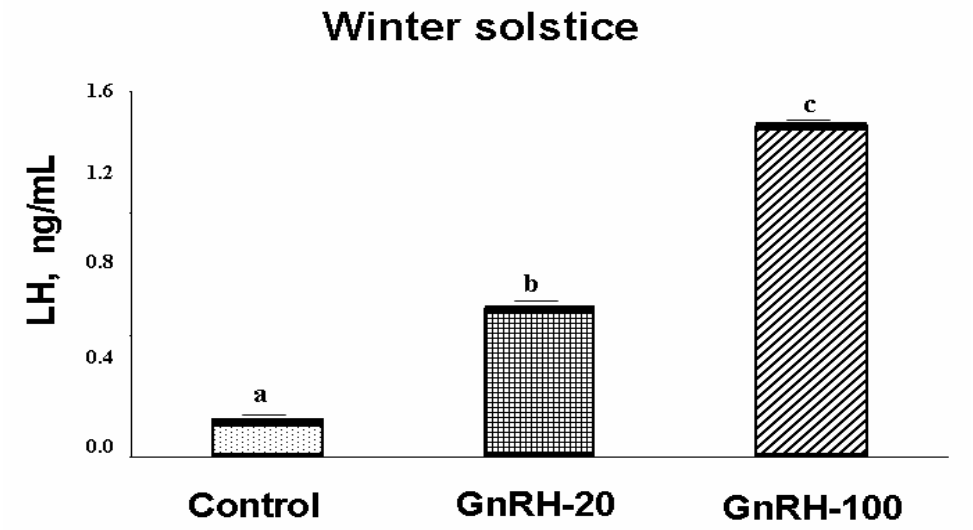

Figure 9. Mean serum concentrations of LH (ng/ml ± SEM) in control mares and mares in the GnRH-20 and GnRH-100 groups at the winter solstice. Means with different superscripts (a,b,c) differed ($P < 0.001$

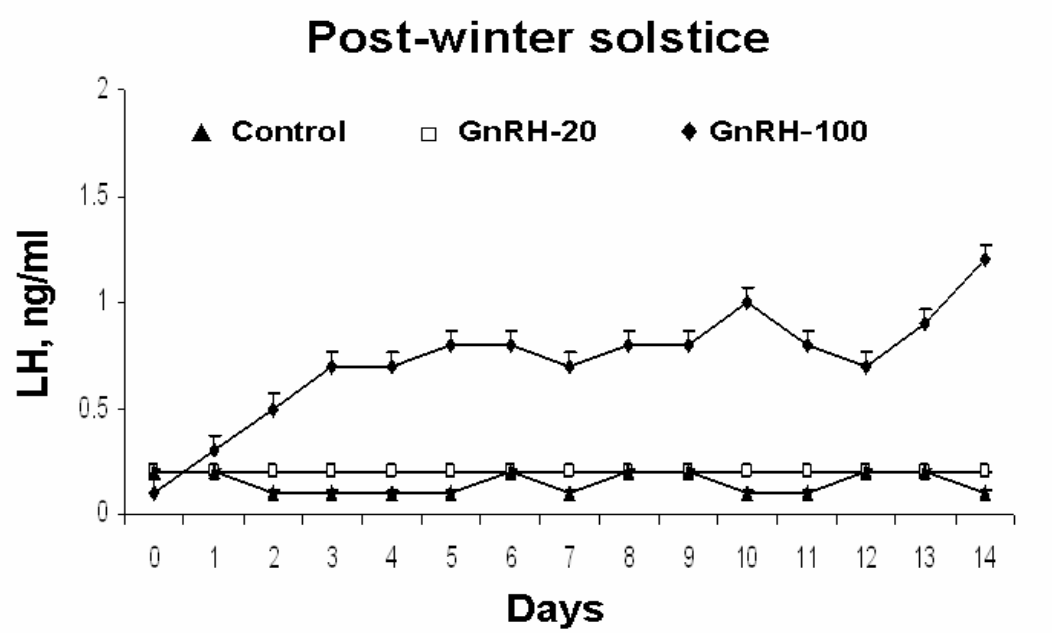

Figure 10. Daily mean serum concentrations of LH (ng/ml ± SEM) in control mares and mares in the GnRH-20 and GnRH-100 groups after the winter solstice (February 15 through 29).

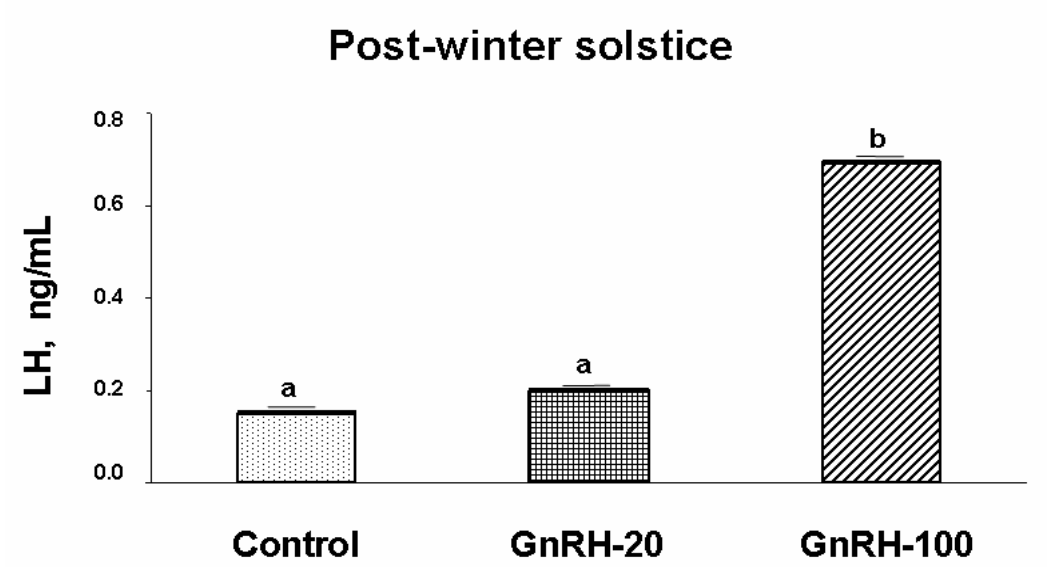

Figure 11. Mean serum concentrations of LH (ng/ml ± SEM) in control mares and mares in the GnRH-20 and GnRH-100 groups after the winter solstice. Means with different subscript (a, b) differed ($P < 0.001$).

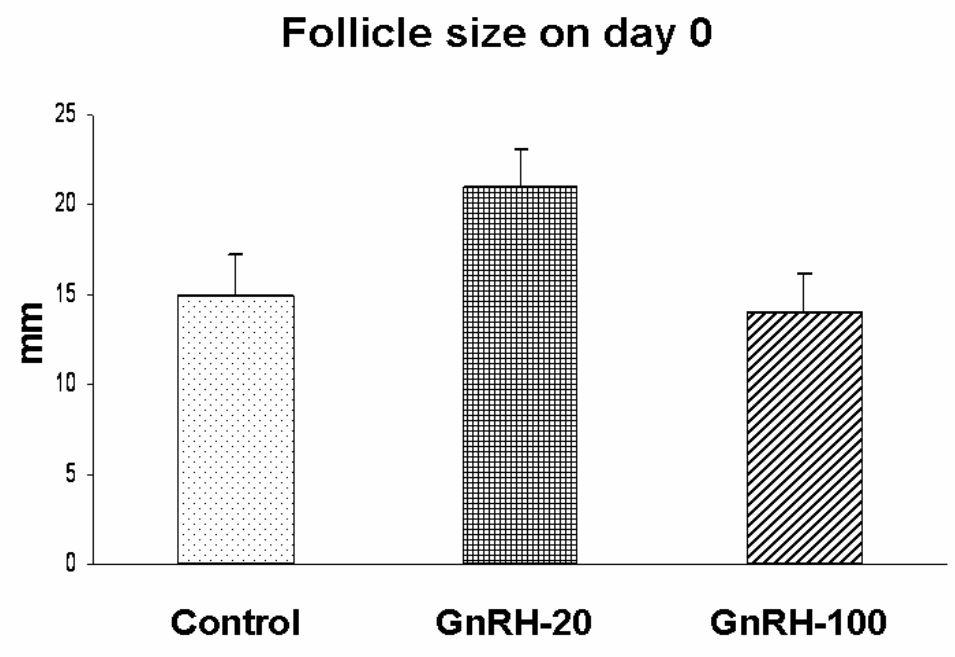

Figure 12. Mean follicle size on day 0 of experiment 2 (± SEM). Mean follicle size did not differ between groups on day 0 of the experiment.

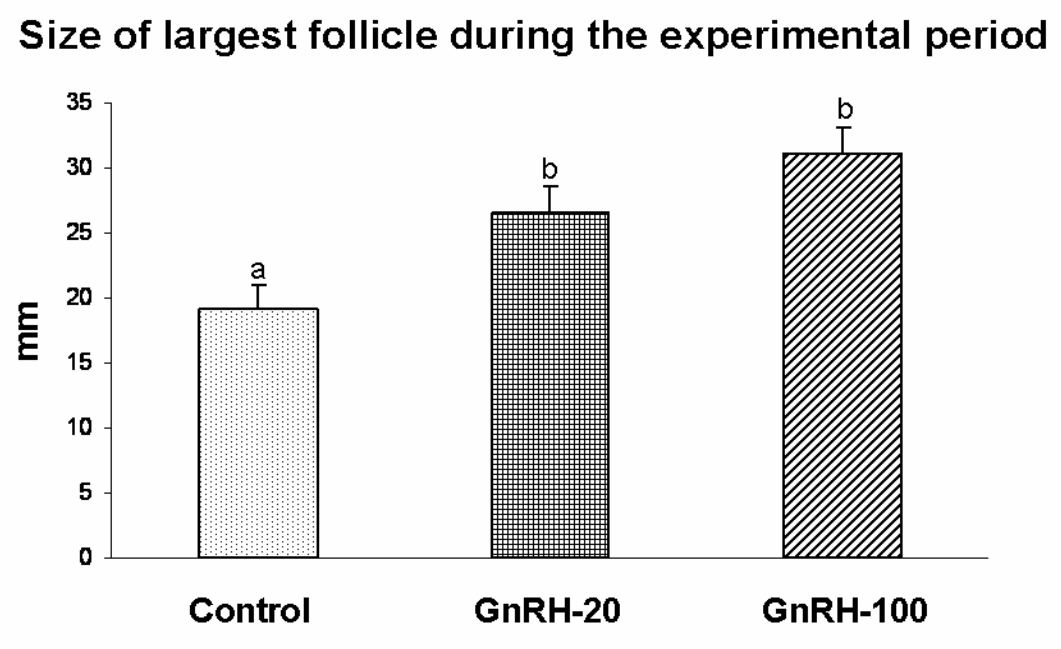

Figure 13. Mean size of largest follicle of experiment 2 (± SEM). Means with different suscripst (a, b) differed. ($P < 0.02$

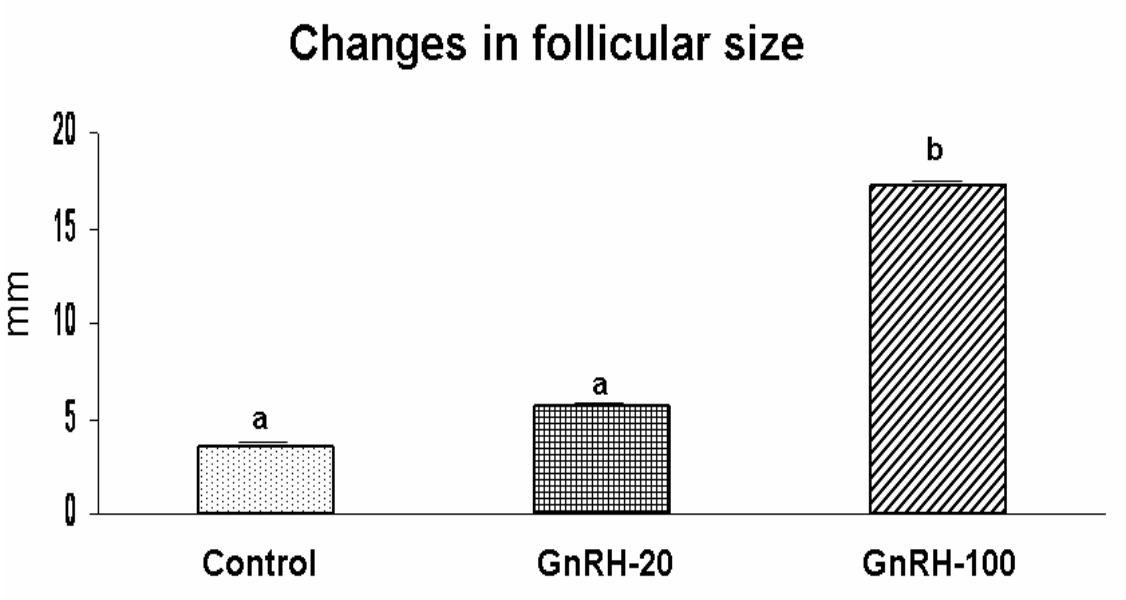

Figure 14**.** Mean changes in follicle size from day 0 of the experiment to largest follicle obtained during the experimental period. (± SEM). Means with different superscripts (a, b) differed. (P< 0.001).

Table 2. Frequency of ovulation near and after the winter solstice.

Treatment	Season	
	Winter solstice	Post-winter solstice
Control	0/5	0/5
GnRH-20	1/5	0/5
GnRH-100	4/5*	2/5*

*Frequency of ovulation differed. (P < 0.007).

3.3.2.4 Estrous behavior

Season did not have an effect on the frequency of occurrence of estrus. Only the GnRH-100 dose increased the frequency of estrus behavior during the experiment (P< 0.003). However, during the post-winter solstice period, one mare out of the GnRH-20

and one mare out of the GnRH-100 group showed estrous behavior but did not ovulate (Table 3).

Table 3. Frequency of estrus behavior near and after the winter solstice.

Treatment	Season	
	Winter solstice	Post-winter solstice
Control	0/5	0/5
GnRH-20	1/5	1/5
GnRH-100	4/5*	3/5*

*Frequency of estrus behavior differed. ($P < 0.003$).

3.4 Discussion

Results of studies reported herein indicate that continuous administration of GnRH stimulates secretion of LH and maintains its episodic release in mares. Furthermore, continuous GnRH stimulation in anovulatory mares appears to be less effective two months after the winter solstice than during the period near the winter solstice. However, larger doses of GnRH (100 μg/h) stimulated LH secretion, follicular development and ovulation at both times of the year.

In the current experiments, we observed a pulsatile pattern of LH release during both the luteal and follicular phases of the estrous cycle utilizing the ICS cannulation technique. An earlier study reported that the majority of pulses of LH observed in pituitary venous effluent of the ICS are not observed in the peripheral circulation [46]. This demonstrates the benefits of accessing the ICS blood for determinations of episodes of LH release. During the luteal phase, when P4 is elevated, pulse frequency is depressed relative to the follicular phase due to negative feedback at the hypothalamic level [46].

Moreover, during the follicular phase, estradiol produced by the growing follicles stimulates LH synthesis and release [28].

In our experiment, observations of high-frequency, short-duration secretory episodes during the follicular phase, and low-frequency long-duration secretory episodes during the luteal phase of control mares, are in agreement with those reported previously [46,93]. Moreover, similar, to our results, Silvia et al. [46] described two distinct types of pulses during the luteal phase: 1) low amplitude-short duration and 2) high amplitude-long duration. In our study, we found short duration secretory episodes that were seen on the descending side of the long duration episodes in the control mares. Similarly, Irvine and Alexander [93] described the pattern of LH during the luteal phase as concurrent, large amplitude, prolonged episodes that appeared to be the summation of a train of pulses. These observations might explain the long duration of the LH secretory episodes during the luteal phase observed in the current study.

Alternatively, the long-duration episodes observed during the luteal phase of control mares might be attributed to variation in the secretory forms of LH during the estrous cycle that have been described in different species: horses [94,95,96], pigs [97], sheep [98, 99,100], cattle [101,102], goats [103] and birds [104]. In cattle, this polymorphism occurs due to variations in the structure of the oligosaccharides within the LH molecule which affect its physicochemical characteristics and the biological and immunological activities of the hormone [105]. Such variations might change the half-life of the LH molecule, creating a long-lasting secretory episode.

Continuous infusion of GnRH (100 µg/h) during the breeding seasons not only stimulated an increase in concentrations of LH but also increased the number of secretory

episodes during both phases of the estrous cycle. In rodents, humans, and sheep, exogenous administration of GnRH must be pulsatile, matching the typical pattern of endogenous GnRH release, in order to provide normal physiological stimulation of anterior pituitary gonadotropes [106,107,108]. Therefore, continuous administration of GnRH causes a reduction in LH release in these species, due to GnRH receptor desensitization or down-regulation [109,110]. When goats, sheep, cattle and monkeys are stimulated with exogenous pulses of GnRH, corresponding pulses of LH secretion are observed [9,111,112,113]. When pituitary cells from rats are cultured *in vitro,* they display refractoriness to constant perfusion of GnRH [114,115,116], but continue to respond indefinitely to pulses of GnRH [116]. This differential response occurs as a consequence of the rapid loss from cell membrane of GnRH receptors during continuous treatment [116]. This seems not to be the case in horses. Several studies have provided evidence suggesting a resistance to GnRH receptor down-regulation in this species [9,117,118]. The equine GnRH receptor differs by 5 amino acids (Ser^{17}, Ala^{26}, His^{61}, Asn^{69}, and Phe^{226}) from GnRH receptors in other species in which the sequence is known. These amino acids reside within the N-terminus, the first intracellular loop and the fifth transmembrane domain. Additionally, similar to other mammalian GnRH receptors, but unlike many G-protein-associated membrane receptors, the equine GnRH receptor has no intracellular carboxy-terminal tail [74]. Cell surface binding does not decline in response to continuous GnRH exposure in the horse. Instead, cell surface binding increases over a 2-h period, indicating that the equine pituitary cell has the ability to provide additional GnRH receptors to the cell surface in the face of continuous ligand stimulation. This is coupled with an apparent slow rate of endocytosis of occupied receptors [74]. These

differences might account for the ability of the equine GnRH receptor to resist desensitization when exposed to continuous GnRH.

Importantly, the release of LH in mares treated continuously with GnRH remained episodic, and this episodic pattern of LH release seems critical for gonadal function in other species. Ovine ovaries infused constantly with LH produce only a transitory rise in P4 secretion, [119]. In male rats, prolonged exposure to constant LH or hCG impaired steroid secretion from testicular tissue [120,121]. Therefore, biological activity of LH at the gonadal level seems to require intermittent stimuli, which appear to be maintained even during continuous GnRH exposure in the mare.

The increase in frequency of secretory episodes of LH during the luteal phase in GnRH-treated mares compare to control could be attributed to an interaction of increased basal concentrations of GnRH induced by exogenous GnRH treatment with endogenous low amplitude but high frequency GnRH pulses. With the elevation in baseline, low amplitude pulses of GnRH that would not generate a detectable episode of LH release could then reach the threshold necessary for stimulation of LH release. This theory is supported by work with growth-restricted lambs, in which there is a central inhibition of GnRH activity [122]. Undernourished, hypogonadotropic ewe lambs manifest there is a decrease in both frequency and amplitude of GnRH pulses. These results in disruption of high concordance between GnRH and LH pulses; small, low-amplitude pulses of GnRH do not produce concurrent pulses of LH [122].

Alternatively, GnRH may act upon GnRH neurons to stimulate GnRH release. Evidence for synaptic specializations between GnRH-containing terminals and GnRH neurons exists [123], suggesting that GnRH may modulate GnRH neuron activity.

Moreover, GnRH-1 receptor mRNA has been identified in subpopulations of GnRH neurons [124]. Therefore, it is possible that exogenous GnRH stimulates endogenous release of GnRH, potentially increasing the amplitude of pulses, which in turn stimulates the release of LH.

In Experiment 1, mean serum concentrations of P4 during the luteal phase increased in GnRH-treated mares compared to controls. These results differ from a previous study in which mares were treated with continuous GnRH for 13 h in mares that were on days 10 to 12 after ovulation. In that study, no differences in P4 concentrations were observed between GnRH-treated and control mares [9]. However, Silvia et al. [46] reported increases in P4 concentrations at four and eight h after a bolus injection of 2 µg GnRH/kg body weight on days five to six after ovulation. In that study, similar to ours, mares were treated with exogenous GnRH within days five and six after ovulation when P4 reaches its maximum plasma concentrations [125]. Therefore, variation in days of treatment within the estrous cycle (early vs. late luteal phase) may explain the conflicting results of these experiments. In support of these observations, the cross-sectional area of the CL increases from day 0 until day 7 coincident with morphological and functional development of the luteal tissue [126,127]. The latter is LH-dependent [128]; thus, increases in pulsatile LH in response to continues GnRH may have supported enhanced development of the corpus luteum at the time of maximal luteal growth response. Moreover, during the follicular phase (36 h after PGF2α administration), mean progesterone concentrations were higher for the GnRH treated mares than controls which was expected since progesterone concentrations were more elevated for this group before PGF2α injection.

During the non-breeding season, continuous and pulsatile GnRH treatments have both been used to stimulate secretion of LH and, consequently, follicular development and ovulation [6,7,10,69]. Most of these studies have been performed after the winter solstice. There is only one study in which low-dose infusion of GnRH (2.5–5.0 μg/h) was applied before the winter solstice (October) and continued through March. These low doses were unable to prevent the onset of seasonal anovulation in the fall or to advance the timing of ovulation in the spring, even though similar doses were previously shown to be effective in about 80% of persistently anovulatory mares during the natural breeding season [12]. We hypothesized that the different responses noted between the seasons is that gonadotropes may be less sensitive to GnRH stimulation in early anestrus compared to late anestrus (before compared to after the winter solstice). In Experiment 2 of the present studies, the anterior pituitary responded to continuous administration of GnRH at a dose of 20 μg/h in December, but not two months later, in late February. Previously, this dose has been used effectively during spring transition to stimulate secretion of LH and ovulation [6,10], but success was greater with pulsatile rather than continuous administration. Becker and Johnson [6] treated anovulatory mares with either pulsatile or continuous GnRH at a rate of 20 μg/h during late non-breeding season. Mares receiving the pulsatile infusion ovulated on average 2.8 days earlier than the mares receiving continuous infusion even though all mares ovulated in both groups. Although the equine anterior pituitary responds to continuous GnRH treatments, pulsatile administration still appears to provide the optimal biological signal [6,10], similar to the situation in other mammals. Silvia et al. [126] provided evidence to indicate that the magnitude of LH release in response to a given amount of GnRH, either naturally

(endogenous) or induced (exogenous), is principally a function of the amount of LH present in the pituitary [126]. Therefore, it is likely that the amount of LH stored in the pituitary is greater early in anestrus than later. Furthermore, our findings suggest that that the seasonal revitalization of LH production and secretion by the pituitary gland is a relatively slow process [126]. This is supported by the observation that when anestrous mares are exposed to an artificial stimulatory photoperiod, the minimum interval to first ovulation varies from two to three months [67,129,130]. In an earlier report [13], we found that the profound reduction in synthesis and secretion of anterior pituitary LH associated with the seasonal anovulatory period may not be due to a reduction in the frequency and amplitude of GnRH pulses, nor in the amount of GnRH reaching the anterior pituitary [13]. However, this remains a controversial finding and must be repeated. If confirmed, the basis for reduced LH in the seasonally anovulatory mare may reside at the level of the anterior pituitary (ie, a reduced response gonadotropes to the GnRH signal). Data from avian species [55,56,59,60], supported by preliminary information in the mare, implies that photoperiodic signals governing seasonal breeding are regulated, at least in part, by an inhibitory hormone, gonadotropin-inhibiting hormone (GnIH) that directly affects the ability of gonadotropes to respond to GnRH [unpublished data]. However, additional research will be required in order to determine whether and to what extent GnIH is involved in equine seasonality.

In both early and late anestrus, follicle development and estrous behavior were stimulated in the majority of mares only in the 100 μg/h GnRH treatment (Exp 2). However, one mare in the GnRH-20 group showed estrous behavior, without evidence of an increase in circulating concentrations of LH or follicular growth, in late anestrus.

Unlike other ungulates, mares often exhibit estrous behavior during anestrus. . In fact, even ovariectomized mares may exhibit estrous behavior [28]. Physiologically, such behavior may be due to stimulation by hormones of non-ovarian origin [131]. The adrenal cortex secretes small amounts of androgens, estrogens and progestins that might influence the expression of estrous behavior [131]. When ovariectomized mares were treated with dexamethasone, which suppresses ACTH secretion and the production of adrenal steroids, estrous behavior was eliminated [131]. Although numbers are low, 2/5 mares in the GnRH-20 group exhibited estrus whereas none of 5 control mares did so, suggesting that this low dose was associated with an increase in estrogen production. However, GnRH-100 consistently increased circulating LH, folliculogenesis and frequency of ovulation, thus it is likely that estrous behavior in this group was associated with enhanced ovarian estrogen synthesis.

CHAPTER IV

CONCLUSION

The equine gonadotrope continues to be GnRH-repsonsive in the presence of treatment with continuous high-dose GnRH during both ovulatory and anovulatory seasons, but it seems to be less responsive late compared to early in the anovulatory season. Data herein suggest that the equine hypothalamic-gonadotropic axis is regulated differently than to that of other mammals, since constant infusion of GnRH did not disrupt the normal pattern of LH release. Constant infusion of GnRH increases the frequency of LH secretory episodes during both follicular and luteal phases of the estrous cycle. The mare is more responsive to contstant infusion of GnRH early in the anovulatory season than later. The exact mechanisms regulating seasonality at the hypothalamic and pituitary levels remain to be fully elucidated.

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APPENDIX

PROCEDURES

Equine LH RIA

References:

Davis SL, Riechert LE, Niswender GD. Biol. Reprod. 4:415- (1971)
Echternkamp SE, Bolt DJ, Hawk HW. J. Anim. Sci. 42:893- (1976)
Golter TD, Reeves JJ, O'Mary CC, Arimura A, Schally AV. J. Anim. Sci. 37:123- (1973)
Niswender GD, Riechert LE, Midgley AR, Nalbandov AV. Endocrinology 84:1166- (1969)
Williams GL, Ray DE. J. Anim. Sci. 50:906- (1980)

1. Iodinated Product: Iodination grade eLH (AFP-5130A).

2. Antibody: Anti-equine LH (AFP-240580). Dilution 1:120,000.

3. Standards: Iodination grade eLH (AFP-5130A). Range 0.1 – 20.0 ng/ml.

4. References: eLH added to equine serum

5. RIA Procedure:
   A. Day 1: Begin Assay
      1. NSB – 500 µl of 1% PBS-EW (egg white).
      2. 0 Std – 500 µl of 1% PBS-EW
      3. Stds – 200 µl std + 300 µl of 1% PBS-EW.
      4. Ref – 200 µl ref + 300 µl of 1% PBS-EW.
      5. Unknown – 200 µl sample + 300 µl of 1% PBS-EW.
      6. Pipette 200 µl of PBS-EDTA + 1:400 NRS without primary antibody into NSB tubes only.
      7. Pipette 200 µl of anti-eLH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes.
      8. Pipette 100 µl 125I-eLH (20,000 cpm/100 µl diluted in 1% PBS-EW) to all tubes.
      9. Vortex tubes briefly and incubate for 24 h at 4°C.

B. Day 2: Add Second Antibody

1. Pipette 200 µl of Sheep-anti-rabbit gamma globulin (SARGG; 2nd Ab) diluted in PBS-EDTA without NRS into all tubes except TC tubes.
2. Vortex tubes briefly and incubate for 48-72 h at 4°C.
   72

C. Day 4: Pour Off Assay

1. Add 3 ml ice cold PBS (0.01 M; pH 7.0) to all test tubes except TC tubes.
2. Centrifuge tubes for 1 h at 4°C at 3600 rpm.
3. Decant supernatant.
4. Count radioactivity of each tube using a gamma counter

Progesterone RIA

Single Antibody RIA Kit, Diagnostic Products Corporation, Los Angeles, CA

References:

Jones EJ, Armstrong JD, Harvey RW. J. Anim. Sci. 69:1607 – (1991)
Diagnostic Products Corporation Coat-A-Count Progesterone Kit, Los Angeles, CA
Simpson, R.B., Armstrong, J.D. and Harvey, R.W. J. Anim. Sci. 70: 1478– (1992).

1. Iodinated Product: Iodination grade hP4.

2. Antibody: Anti-human P4 coated tubes.
3. Standards: Human serum with added P4. Range 0.1 – 20.0 ng/ml.

4. Reference: Human standard preparation added to bovine serum.

5. RIA Procedure:

A. Begin and complete assay
1. Pipette in non-coated polypropylene tubes
NSB – 100 µl of 0 Std
2. Pipette in antibody coated tubes
0 Std – 100 µl
Std – 100 µl
Ref – 100 µl
Unknowns – 100 µl
3. Pipette 1 ml of 125I –P4 provided in the kit to all tubes including two Total Count non-coated polypropylene tubes.
4. Vortex tubes briefly and incubate at room temperature for 3 h.
5. Pour off supernatant.
6. Count radioactivity of each tube using a gamma counter

VITA

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Selected Publications:

Velez I.C., Amstalden M., Pack J., and Williams G.L. Temporal concentration patterns of LH in the intercavernous sinus of the luteal and follicular phase mare during continuous treatment with exogenous GnRH. Proceedings American Society of Animal Science, Annual Meeting, 2008

Velez I.C., Amstalden M., Pack J., Williams G.L. Pituitary responsiveness to continuously- administered GnRH before and after the winter solstice in anovulatory mares. Proceedings American Society of Animal Science, Annual Meeting, 2008

Pack, J.D., **Velez, I.C**., Amstalden, M., Williams, G.L., 2008. Synchronizing new follicular wave emergence in Bos indicus-influenced heifers with estradiol benzoate: Role of the magnitude of the acute increase in progesterone. Proceedings American Society of Animal Science, Annual Meeting.

Pack, J.D., **Velez, I.C**., Amstalden, M., Williams, G.L., 2008. Substitution of estradiol benzoate for GnRH in the Select Synch + CIDR protocol with or without temporary calf removal in Bos indicus-influenced cattle. Proceedings American Society of Animal Science, Annual Meeting.